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Environmental Monitoring and Support Lab.-Cincinnati, Ohio

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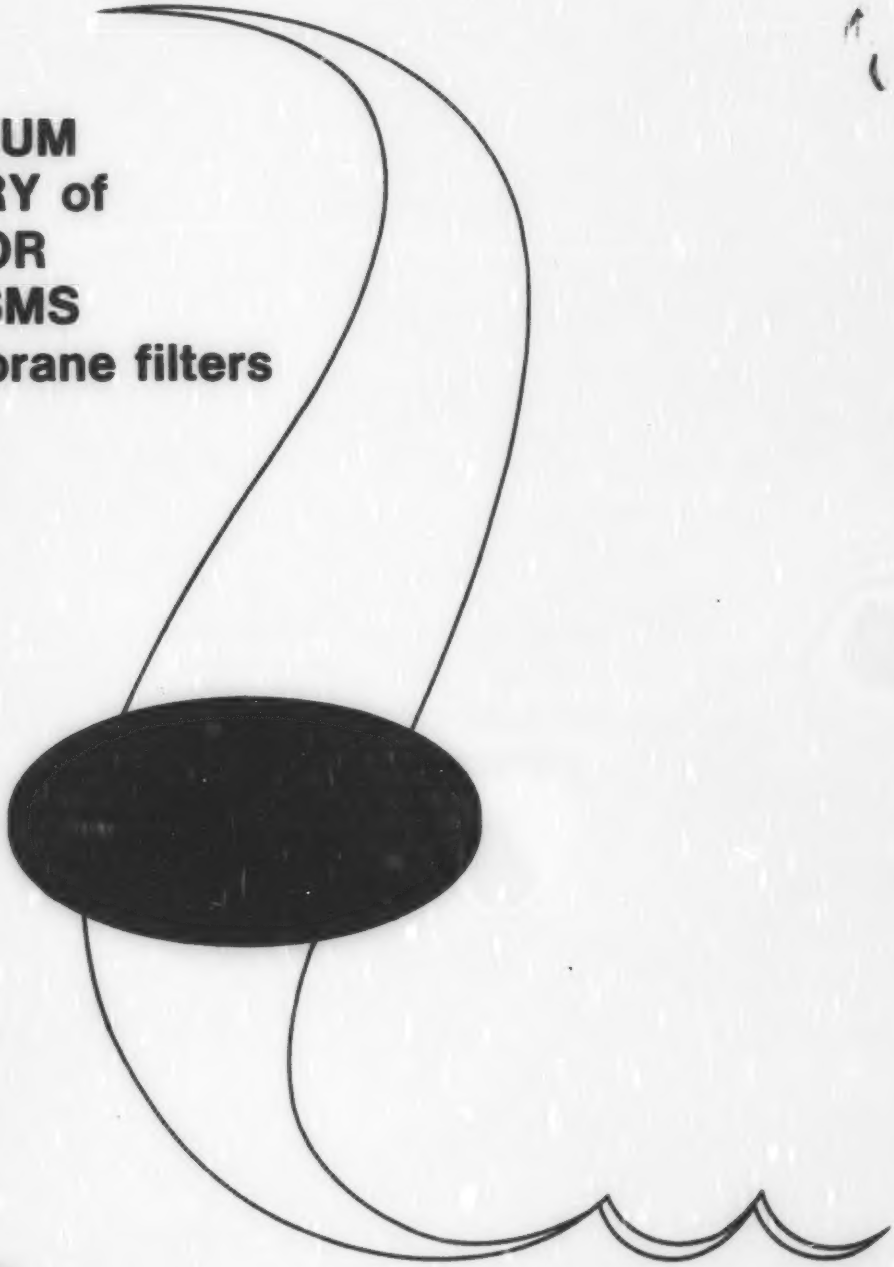
SYMPOSIUM on the RECOVERY of INDICATOR ORGANISMS employing membrane filters



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16. ABSTRACT

The Symposium on the Recovery of Indicator Organisms Employing Membrane Filters brought together users, manufacturers, research scientists and representatives of government agencies to exchange technical information and review the performance of membrane filters for water and wastewater analyses. Problems with the recovery of bacterial indicators had been reported;they were most pronounced in the fecal coliform test. A key question was whether the cause was differences in sample types, membrane filters or the test method employed.

Professionals experienced in water analysis presented relevant field experiences, laboratory data and research findings and discussed problems concerning recovery of organisms stressed or injured by environmental factors. Media, transport phenomena, physical and chemical characteristics of membranes, membrane sterilization methods, incubation temperatures, techniques for comparison of methods, data analysis, and the status of the proposed ASTM methods for evaluating membrane filters were discussed.

Solutions suggested at the Symposium included use of two-step incubation, overlay and/or enrichment techniques and modification of membrane filter structures. Recommendations were made to manufacturers and to users to develop and improve intralaboratory quality control programs, to standardize interlaboratory testing procedures, to participate in these collaborative studies and to generally improve communications among users, manufacturers and standard-setting organizations.

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PROCEEDINGS OF THE SYMPOSIUM ON THE RECOVERY OF INDICATOR ORGANISMS EMPLOYING MEMBRANE FILTERS

EDITED BY

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September 1977

Co-sponsored by:

- The American Society for Testing and Material
- The Environmental Protection Agency

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of waste effluents. The Environmental Monitoring and Support Laboratory-Cincinnati conducts research to:

- Develop and evaluate techniques to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid wastes.
- Investigate methods for the concentration, recovery, and identification of viruses, bacteria and other microorganisms in water. Conduct studies to determine the responses of aquatic organisms to water quality.
- Conduct an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.

This publication of the Environmental Monitoring and Support Laboratory, Cincinnati, entitled:

Symposium on the Recovery of Indicator Organisms Employing Membrane Filters, reports the proceedings of meetings co-sponsored by ASTM and EPA for the specific purpose of solving current analytical problems in microbiology. Such meetings involving the combined expertise of government, academic and industrial laboratories working with the manufacturers have the greatest chance for solutions that will be acceptable to everyone involved in monitoring and controlling pollution in the environment.

Dwight G. Ballinger
Director, EMSL - Cincinnati

ABSTRACT

The *Symposium on the Recovery of Indicator Organisms Employing Membrane Filters* sponsored jointly by the United States Environmental Protection Agency and the American Society for Testing and Materials (Committee D-19 on Water) brought together users, manufacturers, research scientists and representatives of government agencies to exchange technical information and review the performance of membrane filters. Problems had been reported with the recovery of bacterial indicators from water and wastewaters by the membrane filter procedures. They were most pronounced in the fecal coliform test. A key question was whether the cause was differences in sample types, membrane filters or the test method employed.

Professionals experienced in water analysis presented relevant field experiences, laboratory data and research findings and discussed problems concerning recovery of organisms stressed or injured by environmental factors. Media, transport phenomena, physical and chemical characteristics of membranes, membrane sterilization methods, incubation temperatures, techniques for comparison of methods, data analysis, and the status of the proposed ASTM methods for evaluating membrane filters were discussed.

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CONTENTS

Foreword iii

Abstract iv

Acknowledgement vii

List of Attendees. viii

Color Plates. 1

Welcome 5

Summary. 6

Recommendations. 7

Session I Uniform Procedures and Quality Control
Session Chairman – Warren Litsky

The Membrane Filter Dilemma 8
Robert H. Bordner

Performance Variability of Membrane Filter Procedures 12
Edwin E. Geldreich

Quality Control of Membrane Filter Media 20
David Power

Statistical Interpretation of Membrane Filter Bacteria Counts 26
Karl J. Sladek,* Clifford F. Frith and Richard A. Cotton

Effects of Injury on the Recovery of Indicator Organisms on Membrane Filters 34
Alfred W. Hoadley

Effect of Temperature on the Recovery of Fecal Coliforms 42
James B. Hufham

Optimum Membrane Structures for Growth of Fecal Coliform Organisms 46
Karl J. Sladek,* Robert V. Suslavich, Bernard I. Sohn and Fred W. Dawson

Session II Comparision Studies of Membrane Filters
Session Chairman – Phillip E. Greeson

A Comparison of Membrane Filters and Media Used to Recover Coliforms from Water 58
Michael H. Brodsky* and Donald A. Schiemann

Comparison of Membrane Filters in Recovery of Naturally Injured Coliforms 64
John E. Schillinger, Gordon A. McFeters and David G. Stuart*

Efficiency of Coliform Recovery Using Two Brands of Membrane Filters................ 67
Frederick A. Harris* and Carl A. Bailey

Comparison of Membrane Filter Brands for the Recovery of the Coliform Group 73
Alfred P. Dufour* and Victor J. Cabelli

A Comparison of Membrane Filters, Culture Media, Incubation Temperatures, Polluted Water and Escherichia coli Strains in the Fecal Coliform Test.................. 82
Paul J. Glantz**

Session III Modifications to Improve Recovery
Session Chairman – Phillip E. Greeson and Robert H. Bordner

Recovery Characteristics of Bacteria Injured in the Natural Aquatic Environment 98
Gary H. Bissonnette, James J. Jezeski, Gordon A. McFeters* and David G. Stuart

A Layered Membrane Filter Medium for Improved Recovery of Stressed Fecal Coliforms ... 101
Robert E. Rose, Edwin E. Geldreich* and Warren Litsky

Measurement of Fecal Coliform in Estaurine Water.................................. 109
Alanson P. Stevens, Rosario J. Grasso* and John E. Delaney

An Evaluation of Methods for Detecting Coliforms and Fecal Streptococci in Chlorinated Sewage Effluents 113
Shundar D. Lin

The ASTM Proposed Membrane Filter Test Procedure for the Recovery of Fecal Coliforms.. 133
Don W. Davis,* Margareta Jackson and George R. Kinser

Critique on ASTM Test for Recovery of Fecal Coliforms and Proposal for Modified Method... 153
Norman H. Goddard

Summary of Symposium.. 157
Francis Brezenski and John Winter

Final Discussion 162

Final Remarks.. 175
Clifford F. Frith

APPENDIX

Comparison of Membrane Filter Counts and Plate Counts on Heterotrophic and Oil Agar Used to Estimate Populations of Yeast, Fungi and Bacteria 178
J. D. Walker, B.F. Conrad, P.A. Sessman, and R.R. Colwell

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ACKNOWLEDGEMENT

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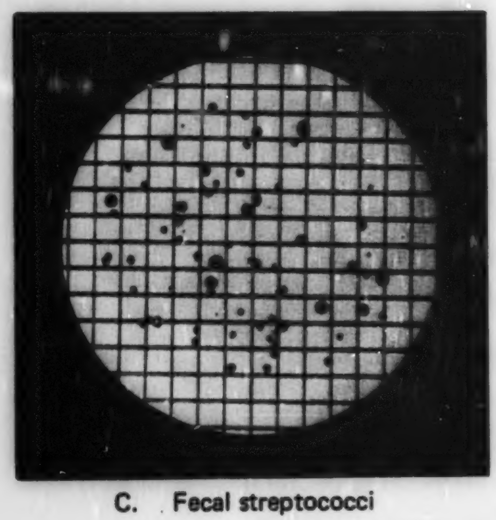
A. Total coliforms

B. Fecal coliforms



C. Fecal streptococci

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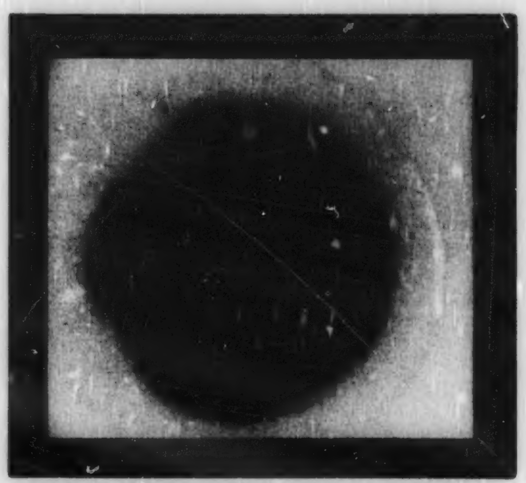
Plate 1. Recovery of Indicator Microorganisms by Membrane Filter Methods.

A. Fecal coliform and non-coliform colonies



B. Fecal coliform colony showing crystalline structure

C. Total coliform sheen colony

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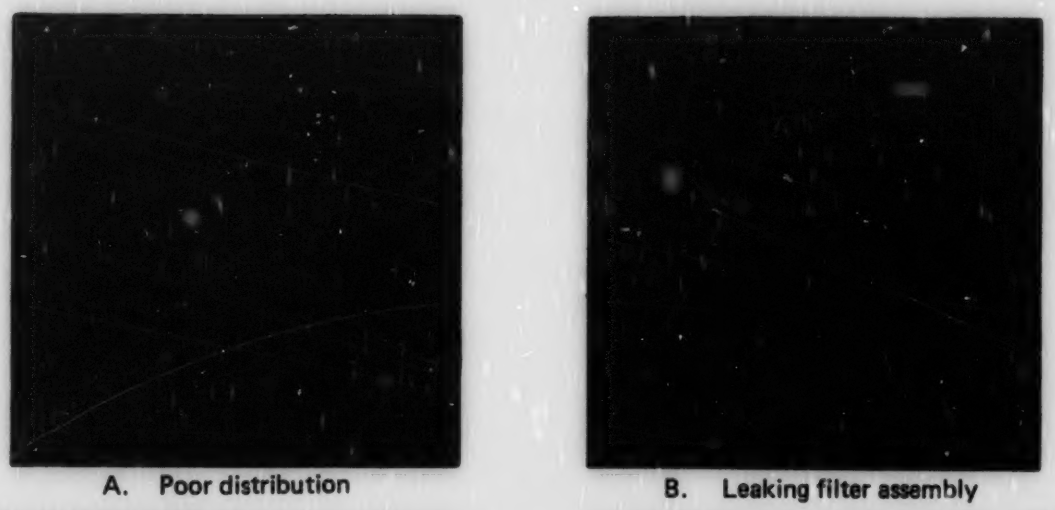
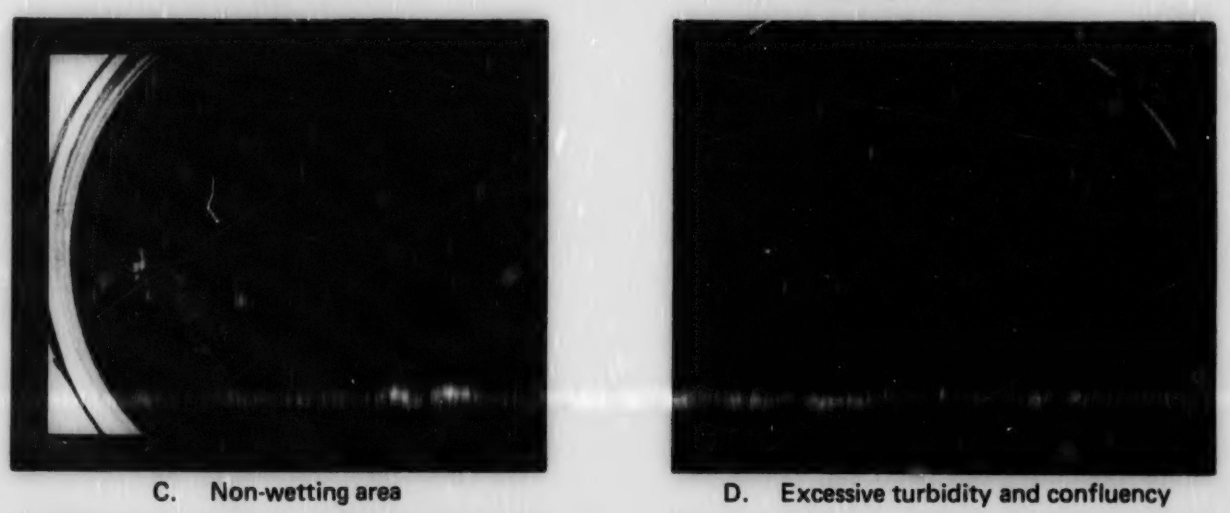
D. Fecal streptococci colonies

Plate 2. Close-ups of Indicator Microorganisms on Membrane Filters.



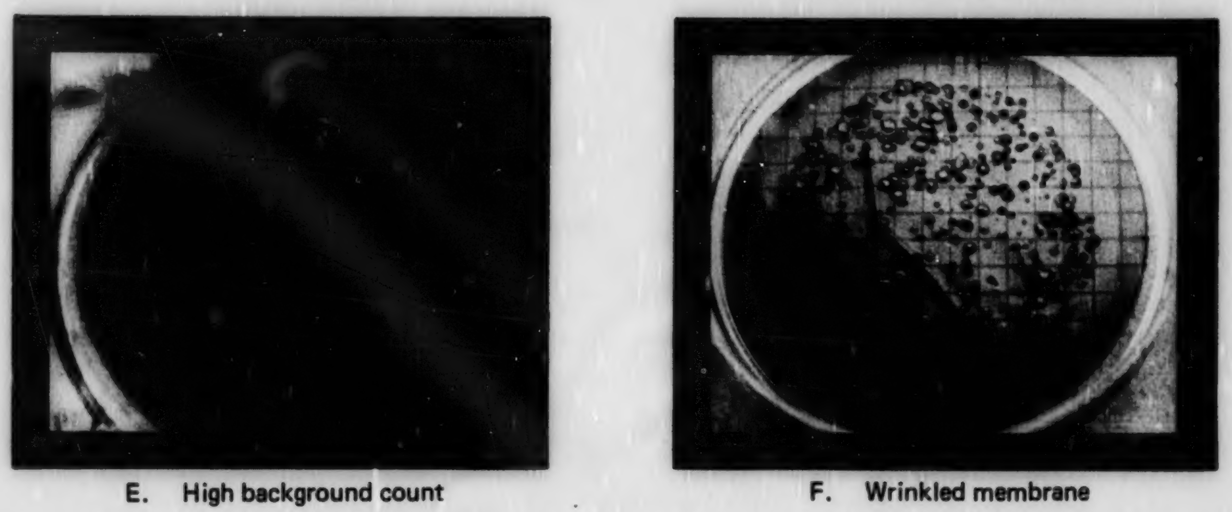
A. Poor distribution

B. Leaking filter assembly



C. Non-wetting area

D. Excessive turbidity and confluency



E. High background count

F. Wrinkled membrane

Plate 3. Problems in Recovery of Indicator Microorganisms as Shown in the Total Coliform Test.

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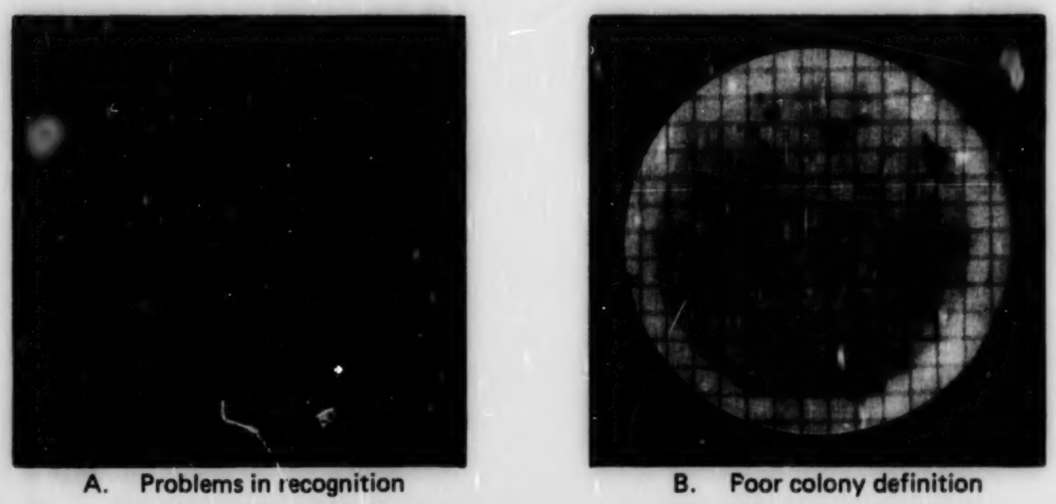
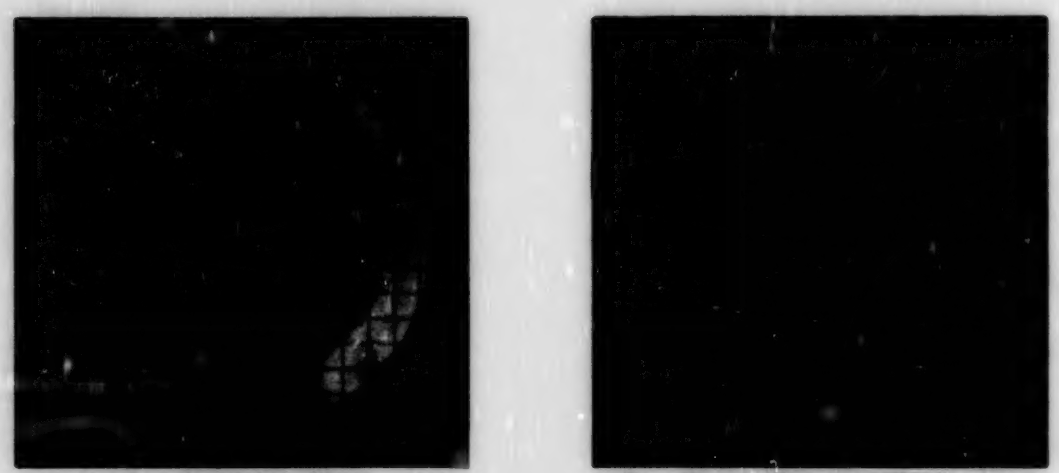
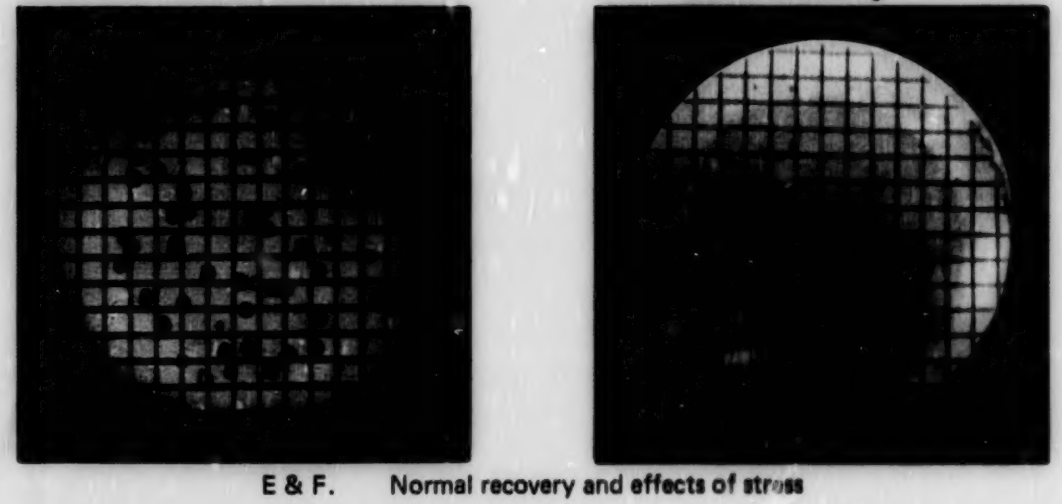


A. Problems in recognition B. Poor colony definition



C. Turbidity effects D. Channelling, poor distribution and overcrowding



E & F. Normal recovery and effects of stress

Plate 4. Problems in Recovery of Indicator Microorganisms as Shown in the Fecal Coliform MF Test.

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WELCOME

Robert H. Bordner

On behalf of the American Society for Testing and Materials and the U.S. Environmental Protection Agency, we warmly welcome you to ASTM Committee D-19 on Water, and to the **Symposium on the Recovery of Indicator Organisms Employing Membrane Filters.** We have estimated the number of attendees to be 105. This large attendance reflects the high interest in the use of membrane filters to recover coliforms and other bacteria from water.

ASTM Committee D-19 has been sojourning to Florida every January for ten years and last year the D-19 meeting attracted about 200 enthusiastic members to Fort Lauderdale. Committee D-19 has grown rapidly from the original subcommittees for organic substances, metals and inorganic constituents in water and now includes many other test areas, such as oil identification, sediment chemistry, automated analyses, and biological methods. This year the total attendance of the D-19 meeting has doubled, reflecting the increasing activity in biological and microbiological monitoring.

The subject of this symposium falls under the Subcommittee D-19.08 on Membrane and Ion Exchange Materials. A task group of this subcommittee was formed to develop test procedures for evaluation of membrane filter materials. It is appropriate that this symposium be held under the sponsorship of ASTM as well as EPA, because of the Society's almost-unique structure that brings together representatives of the manufacturers, users, regulatory agencies and research workers to attack problems of common concern.

SUMMARY

The results of the studies of membranes described in this symposium cannot be compared directly because of non-uniform test conditions. However, we can reach the general conclusion that variable recoveries of coliforms, fecal coliforms and fecal streptococci occur with testing of water and wastewater samples by membrane filtration (MF), further, that low counts result from injury caused by natural stream conditions, chlorination, the elevated incubation temperature of the fecal coliform test and the structure of 0.45 µm membrane filters.

The effects of stress on recovery are confused by the variable and unpredictable low recoveries of indicator organisms from different lots and brands of membrane filters. Because most laboratories are not conducting routine quality control checks on materials, media, equipment and methodology as part of a within-laboratory QC program, are not analyzing enough split or replicate samples, and are not verifying sufficient test results to insure the validity of their data, further discrepancies occur in comparative data.

It was also obvious from discussion that the poor communications which exist between manufacturers and users contribute to the problem.

Proposed solutions to the problem of low recovery of indicator bacteria on MFs were:

1. Incubation of filtered samples on a non-selective medium prior to transfer and incubation on the selective medium.
2. Short term incubation of filtered samples on a selective medium at 35 C prior to incubation at 44.5 C.
3. Short term incubation of filtered sample on a non-selective medium at 35 C prior to transfer and incubation on a selective medium at 44.5 C.
4. Use of an overlay of a non-selective agar on a base layer selective agar for short term incubation at 35C prior to incubation at 44.5C.
5. Use of a larger surface-pore membrane filter in place of the 0.45 µm pore standard filter.

RECOMMENDATIONS

To the ASTM Subcommittees

Develop MF collaborative testing procedures for physical, chemical and microbiological characteristics that include tightly written protocols, which control test variables and include randomization and standardized statistical evaluation.

Develop a collaborative testing program for comparison of methods for indicator organisms.

Develop a uniform test protocol for comparing membranes, media and other test conditions. Until this is done it will be impossible to evaluate and select improved methodology for indicator bacteria.

To Membrane Filter Users

Establish a quality assurance program within the laboratory for supplies, equipment, and analyses. Such control on membranes, media, and test conditions will assure valid data.

Follow standard test protocols developed by EPA/ASTM committees for future within-laboratory or interlaboratory studies.

Support ASTM, EPA and other testing groups in evaluations of improved MF procedures using the standard test protocols.

To Manufacturers

Expand and improve the quality control of membrane filters, media, reagents, and equipment used in MF tests.

Encourage the certification of specific products for water analysis.

Improve communications with users. Provide them with necessary information on pore size, configuration, additives (extractables), membrane materials, manufacturing dates of media, changes of formulations etc.

Establish a voluntary program to notify users about unacceptable lots and recall such products if necessary.

To EPA

Develop a test protocol for the comparison of test methods.

To Researchers

Investigate the physiological basis for environmentally-stressed cells and apply the results to improved MF methods.

Solve the problem of testing chlorinated effluents by MFs.

Define MF specifications for a) a completely inert, or b) highest count membranes.

THE MEMBRANE FILTER DILEMMA

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ABSTRACT

Reported variances in recovery of indicator organisms, symposium objectives and requirements for improvements in membrane filter (MF) procedures are described. The applications of MF methods to water quality control and recent enforcement legislation are reviewed. The importance of quality assurance procedures and the role of The American Society for Testing and Materials in the development of standardization test methods for water are discussed.

INTRODUCTION

The dilemma that we face here today is that for the past year and a half the membrane filter has been the target of serious charges concerning its inability to adequately recover indicator organisms from water and wastewater. Conflicting reports in the literature have documented the differences in recovery among various membrane filter brands. Some laboratories have reported variations in membranes and media from lot to lot. Other investigators have encountered low recoveries when using the membranes to enumerate indicator groups from marine waters and chlorinated effluents, or those containing toxic materials.

The requirements to enforce and monitor water and wastewater standards recently established by the U. S. Environmental Protection Agency have greatly accentuated the need for precise standard procedures and reliable, uniform test materials.

SYMPOSIUM OBJECTIVES

This symposium was organized to focus on the real ability of membrane filters to recover indicator organisms. Much consideration will be given to fecal coliforms, because they are the indicator group of primary concern. Also, recovery problems are intensified at the elevated temperature of the fecal coliform test.

The ultimate objectives of this symposium are to:

1. Identify problem areas and future needs for the use of membrane filters.
2. Review differences in the recovery of microorganisms and investigate the cause of these differences.
3. Determine the factors that affect recovery.
4. Define the type(s) of filters required for water analysis.
5. Develop test procedures for the evaluation of membrane filters that will assure quality control.

THE MEMBRANE FILTER PROBLEM

The membrane filter, first introduced in this country about 25 years ago, has developed over the years into an estimated 5 million dollar a year industry, and is widely accepted by water microbiologists for many different tests. The microbiologist has learned to appreciate the advantages of membrane filter procedures: the rapidity, expediency of direct counts, ease of testing, minimal space and labor requirements, ability to examine large sample volumes and portability for field testing. He must now consider which brand of filter to use or, indeed, whether to use the membrane filter at all to obtain good recovery.

The manufacturers know the needs and concerns of the users, and have taken a close look at their product, conducted their own investigations, and in some cases modified membrane formulations in an effort to improve the filters for water analysis. They are becoming more aware of the importance of good quality control of membrane filter materials.

There are several questions related to the membrane filter dilemma that we should consider during this symposium:

1. What degree of recovery is required? Is the ultimate goal the recovery of all organisms that will grow under one set of test conditions for a given parameter (e.g., specific membrane, medium, and incubation temperature), or is it the recovery of all viable organisms, including attenuated organisms, to produce a result as close as possible to the true count?
2. What type of membrane filter is necessary? Do we need a filter that is completely inert and does not in itself affect growth and recovery, or one that does not contain nutrients, materials or inhibitory materials? Should a filter be considered that enhances recovery and growth by releasing soluble materials which stabilize pH or have a beneficial buffering effect on the medium? Manufacturers point out that such materials are available.
3. Will membranes provide good recovery under prescribed test conditions for one indicator but not provide equally good recovery for other indicators under different conditions? Is it desirable to design a membrane material for the optimal recovery of each specific indicator group?
4. What is the relationship of the membrane to the underlying media? For example, is recovery affected by agar or broth-saturated pad substrates?
5. Does the method of sterilization affect the ability of the membrane to recover organisms, as recently reported?
6. How can better recovery be provided for problem samples such as marine and estuarine waters, chlorinated effluents, and wastewaters containing phenols, metals or other toxic compounds?
7. What product specifications or limits are critical for membrane filter materials?
8. What is the real shelf life of the membrane filter?
9. What quality assurance does the manufacturer perform?
10. What quality control procedures must the laboratory carry out on membrane filters as well as other materials, such as the media, reagents, distilled water and other supplies?
11. What are the microbiological and chemical characteristics of membrane filter materials for which ASTM subcommittees should develop practical test procedures, for example, recovery, inhibitory effects, retention and extractables?
12. What statistical measurements should be used uniformly so that membrane filter tests can be compared fairly among laboratories?

USES OF MICROBIOLOGICAL ANALYSES

Microbiological analyses of water and wastewater are conducted in order to:

1. Assure the quality of potable water at the water treatment plant and in the distribution system, raw water sources, ground water, and bottled water.
2. Determine the quality of water for recreational, argicultural, irrigation, industrial, shellfish-raising, and other uses.
3. Investigate the quality of municipal and industrial wastewaters, and the effectiveness of treatment.
4. Plan and develop water resources.
5. Perform in-plant studies.
6. Identify the source or trace the disposal of bacterial pollutants.

7. Carry out research investigations.

8. Monitor and enforce established standards for wastewater effluents and receiving streams.

WATER AND WASTEWATER STANDARDS AND CRITERIA

The requirement for monitoring and enforcing water quality standards is one of the most compelling reasons for developing standard methods and uniform materials.

The standards for potable water quality were provided for in the Safe Drinking Water Act (Public Law 93-523), dated December 16, 1974 (1). Within 90 days after enactment, the maximum allowable levels of constituents should be published in the Federal Register as EPA standards. The MPN or MF procedure may be used to monitor these limits. For the membrane filter technique, the quality limit is one total coliform per 100 ml and the action limit is more than 4 total coliforms per 100 ml. The minimum action required is immediate repeat sampling. The volume sampled by the MF technique must be 100 ml. The minimum number of samples collected each month is based on the population served by the supply. A standard plate count incubated at 35 C for 48 hours is recommended.

The suggested criteria for natural waters were spelled out in the **Water Quality Criteria, 1972** (2). This updated volume of the original Water Quality Criteria published in 1968 (3), was developed for EPA by the National Science Foundation. A supplemental document will be forthcoming to support the enforcement of these criteria. The recommended criterion for recreational water is 200 fecal coliforms per 100 ml. The criteria for shellfish-raising waters are 70 total coliforms and 14 fecal coliforms per 100 ml.

The Federal Water Pollution Control Act Amendments of 1972 (Public Law 92-500) (4) established guidelines for the levels of constituents in municipal and industrial effluents. The monitoring of these standards, under the new national permit system, is entitled National Pollutant Discharge Elimination System, (NPDES). The minimum level of effluent quality attainable by secondary treatment for fecal coliforms was described in the **Federal Register**, August 17, 1973 (5). The Act states that the geometric mean of the fecal coliform value for effluent samples collected over a period of 30 consecutive days shall not exceed 200 per 100 ml for fecal coliforms. The geometric mean for 7 consecutive days shall not exceed 400 per 100 ml.

The microbiological guideline for industrial effluents from the food processing, textile, feedlot, meat products, tanning and sugar processing industries is a maximum fecal coliform value not to exceed 400 counts per 100 ml at any time.

QUALITY ASSURANCE

One of the primary responsibilities of the microbiology laboratory is the adoption of a formal quality control program to assure the reliability and validity of laboratory and field data. The quality assurance program includes the systematic practice of accepted sampling and analytical procedures described in **Standard Methods** or in the forthcoming EPA manual on microbiological methods. Quality assurance by trained laboratory and field personnel and application of good quality control over materials, equipment, instrumentation analyses and the resultant data are required.

Assured validity of results is particularly important if the data are used in court, in the exchange or compilation of data from other laboratories, or entered in a data storage bank for other users.

MEMBRANE FILTER SPECIFICATIONS

In 1965 the Department of Defense, Office of Medical Materiel, developed a detailed set of interim specifications to control the quality of membrane filter materials. Test procedures were described for characteristics such as recovery, toxicity, retention, flow rate, porosity, pore size, and extractables. These specifications have been superceded by Military Specifications dated September, 1973 (6).

DEVELOPMENT OF ASTM TEST PROCEDURES

The American Society for Testing and Materials is directly concerned with the quality control of materials such as membrane filters because of its fundamental interest in the development of standards of characteristics and performance of materials and the promotion of this knowledge.

ASTM provides a unique system whereby experts from several fields can get together and develop, evaluate and approve test procedures by group concensus. ASTM publishes the procedures using an established protocol and a standard format. If an evaluation process is required to characterize materials available from various manufacturers, to determine that materials are uniform, or to know that purchase from random sources will give acceptable results, the mechanism is available through ASTM. This is the real reason for this symposium. The test procedures for membrane filters are being developed through this system.

To develop a test procedure, a task group chairman solicits proposed methods. These draft methods are circulated for review until the chairman is satisfied that he has a consensus of agreement on a proposed test procedure. This procedure is then tested by volunteer laboratories in a round robin procedure if it is amenable to this type of collaborative testing.

Under ASTM rules, it is necessary to have supporting data to show that the method is acceptable. Statistically significant results must demonstrate the desired precision, and, if possible, accuracy. The proposed test procedure is then submitted to the main D-19 committee for approval by ballot. If the procedure is approved and negative votes, if any, have been resolved, the method is published in the ASTM manual as an official procedure with tentative status.

ASTM TASK GROUP ON MEMBRANE FILTERS

The present subcommittee task groups on microbiological properties and physical-chemical characteristics of membranes originated in June, 1971 with subcommittee D-19.08, which was organized to investigate test procedures for membranes used for separating processes such as ion exchange, electrodialysis, reverse osmosis, and ultrafiltration. At the initial meeting of this group much confusion arose over the basic question, "What is a membrane filter?" It soon became apparent that the need for controlled pore size filters varied with the use. Because membrane characteristics are related to their application, subsections were developed by use under specific categories.

The present session of this particular subcommittee that is developing test procedures for membrane filter materials used in water analyses is the third since the organizational meeting in June, 1973. The development of test methods has proceeded through the early discussion and review stages, but not without growing pains. Test procedures for recovery and inhibitory effects were submitted to the subcommittee. Preliminary round robin tests were carried out on 6 brands of filters by 10 volunteer water laboratories. The statistical results indicate a need for a better test procedure. A detailed report on this procedure and the round robin test results will be presented later at this symposium.

The present status of ASTM test procedures for the microbiological properties of membrane filter materials is: 1. A preliminary procedure for recovery has been developed but requires modification. 2. A modified test procedure for inhibitory effects is ready for resubmission to the subcommittee. 3. A proposed procedure for retention has been prepared for task group discussion.

The conflicting and confusing reports on the recovery of indicator organisms and the lack of a test procedure for recovery precipitated the organization of this symposium to provide a forum for the in-depth examination of these problems.

REFERENCES

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PERFORMANCE VARIABILITY OF MEMBRANE FILTER PROCEDURES

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ABSTRACT

Performance variability in membrane filter procedures can be traced to a variety of factors including variations in membrane filter (MF) manufacture, absorbent pad impurities, MF sterilization procedures, commercial media inconsistencies, and technician knowledge, skill and judgement in applying MF procedures to water analyses. The acceptability of special MF devices in terms of data reliability is also reviewed.

INTRODUCTION

Many of the problems to be identified in this presentation on the membrane filter dilemma can be traced to technological developments that have failed to recognize the critical requirements associated with microbiological applications. As a consequence, a variety of factors contribute to the overall problem whose magnitude threatens the future of this valuable microbiological tool. Prior to cataloguing these factors, a brief historical resume should place these issues in better perspective and hopefully point to directions that both the manufacturers and laboratories must take to restore confidence in membrane filter procedures.

Historical Development of the Membrane Filter

The initial attempt to develop an artificial membrane as a substitute for those found in nature has been credited to Fick in 1855 (1-3). However, Fick experienced difficulties in using these fragile collodion membranes and eventually abandoned the idea. Once the conceptual design of a collodion sac for dialysis became apparent, numerous applications of this membrane form were reported during the period of 1893 and 1905 (1). Soon it was discovered that the mixture and concentration of alcohol-ether and glacial acetic acid solvent systems would change the porosity in the nitrocellulose during evaporation and that the resulting permeability could be preserved by placing the forming membranes in water before solvent evaporation was completed (4-8). Glycerol, soluble in the alcohol-ether mixture but insoluble in nitrocellulose, was found to increase membrane permeability (9). Improved flexibility of the finished membrane was achieved by the addition of a small concentration of castor oil. From these pioneering investigations it became evident as early as 1915 that: a) the control of porosity was the key to the successful development of nitro-cellulose membranes; b) reproducibility of pore size was easier in membrane sheets than in the production of a sac configuration and; c) there was a need for careful control of production methods and exact concentration of ingredients used in the preparation of membranes (10).

By 1916 the usefulness of a nitro-cellulose membrane as a tool in bacteriology had been recognized. Removal of bacteria from any reasonable quantity of fluid and cultivation of the microorganisms in place on a membrane surface had long been a goal for many investigators. However, the usefulness of such membranes was extremely restricted because their preparation was difficult and the product was of uncertain quality and porosity. Zsigmondy and Beckman were the first to develop a method for the preparation of a membrane that could readily be adapted to commercial production and they were issued a United States patent in 1922 based on an application submitted in 1919 (11-14). This procedure consisted of dissolving nitro-cellulose or nitro-cellulose acetate in a mixture of acetone and glacial acetic acid, pouring the solution on a glass plate in a thin layer; allowing the volatile solvents to evaporate at 18 C in a 60 percent relative humidity; and washing the finished membrane in water. Pores of a specific size range were produced by controlling the concentration of nitro-cellulose in the basic solution, composition of solvent mixture and relative humidity during solvent evaporation. Increasing the percent of water in the solvent mixture or elevating the percent relative humidity during the drying process resulted in larger pore sizes.

The commercial manufacturing technology for membrane filters remained basically unaltered from the Zsigmondy process until the post World War II period. From a review of German military intelligence gained during a scientific reconnaissance after the war, Dr. Alexander Goetz prepared a complete report on the manufacturing process, characteristic properties and bacteriological applications of the Zsigmondy membrane filter (15). Research into production techniques for the U.S. Army Chemical Corps led Dr. Goetz to develop a membrane filter that did not require storage in water, to incorporate a grid imprint, and to design an associated apparatus for use in filtering water. This improved processing was then contracted to the Lovell Chemical Corporation which later organized the Millipore Filter Corporation for American production of the improved filter made from domestic materials. Numerous refinements relative to automation of the process have since been made to improve the uniformity and quality of the product in a competitive market that includes several other American membrane filter manufacturers: Gelman, Schleicher and Schuell, Nuclepore, Helena, and Johns-Manville. Among the foreign manufacturers, Oxoid (British) and Sartorius (German) are the most available membranes in this country.

Application of the nitro-cellulose membrane developed simultanously with the technological advances in membrane filter manufacture. However, application to bacteriological procedures was hindered in early years by the uncertain quality and inadequate supply of the material. In 1919, a nitro-cellulose filter was first reported to recover organisms of tuberculosis from urine (16). Apparently, the first attempts to culture microorganisms on membrane filters were done in Russia and Germany during the 1930's (17-21). Dr. Mueller (Hygienic Institute, Hamburg) adapted these techniques to the urgent needs for an adequate monitoring of public water supplies and for emergency situations resulting from the war devastations of Germany in the period of 1943 to 1945. Routine analysis of potable waters for coliforms was accomplished by placing the membrane, after filtration, on a substrate of seven filter papers saturated with Endo broth (20). Mueller was also successful with this type of procedure in her investigation of an epidemic of typhoid fever occurring in Hamburg (21). Samples were filtered through a nitro-cellulose membrane filter which was then placed on a modified bismuth sulfite medium for cultivation of typhoid organisms. The interest in membrane filter techniques for the bacteriological examination of water became widespread throughout the English speaking world following studies in the nineteen-fifties by Clark et al. (22,23) and Goetz et al (24, 25) in the United States and Taylor and Burman (26, 27) in Great Britain.

Analysis of data available to the U.S. EPA laboratory evaluation program indicates that state health, state environmental, city-county health, municipal water treatment and private laboratories are examining approximately 3.5 million samples annually from this nation's public and private supplies and in gathering and monitoring data on natural waters relative to state and federal standards for a variety of water quality uses (28). An estimated one million additional samples are analyzed by local laboratories in quality control monitoring of industrial and municipal waste discharges as required in the National Pollution Discharge Elimination System. Of course, not all of these analyses involve the membrane filter procedure but national statistics suggest 52.1% of all laboratories currently involved in the nationwide laboratory evaluation program are using the membrane filter procedure on a variety of water samples estimated to be 1.8 million analyses per year. Thus, there is a substantial amount of monitoring data being developed from membrane filter procedures which should be reliably measuring water quality.

Membrane Filter Quality for Microbiology

Commercial brands of membrane filters may vary in performance as a result of manufacturing technology, materials, and degree of quality control exercised. For microbiological applications, there must be a complete retention of organisms on or near the surface of a non toxic, inert matrix which permits a continuous contact with nutrients from a medium held in a substrate below the membrane. These basic conditions place demanding requirements on the quality of every membrane used in the laboratory. Basic difficulties encountered with membrane filters generally relate to pore distribution, hydrophobic filter areas, grid line ink restrictions, membrane materials, sterilization practices, and poor storage characteristics that cause increased filter brittleness and surface warping (29-39).

Membrane filter pores should be uniformly distributed and have a diameter of 0.45 micron (± 0.02 micron) for routine bacteriological techniques. Pores of some commercial lots of membrane filters have been found to be so small in some areas of the filter that serious local reduction in the flow rate occurs. The filter should be free of visible non-porous areas which prevent the diffusion of nutrients to the upper surface of the membrane. Any bacterial cells entrapped on such surfaces will not develop into visible colonies for lack of nutrients. When M-Endo is used in a test of diffusibility, non-wetting areas on the filter will remain white and dry. Such observations should not be confused with air bubbles, which can be removed by reseating the membrane over the medium-saturated pad or agar base. At the other extreme, pores larger than 0.7 micron will not retain organisms associated with indicator groups. For complete bacterial separation from liquids, membrane filter porosity of 0.22 micron is required to insure retention of the smallest bacteria through physical impingement or electro-static entrapment.

The ink used to imprint the grid system on the membrane filter should be non-toxic to all bacteria cultivated on the filter' surface (30). Some inks have been found to be bacteriostatic or bactericidal. Such effects can be recognized through restrictive colony development adjacent to the imprinted lines. These growth restrictions may not only be caused by inhibition from toxic inks but also from thick ink imprints that "wall-in" grid squares and by hydrophobic inks which prevent nutrient diffusion to sites in the ink imprint. As an additional characteristic, inks selected for grid imprinting should not "bleed" across the membrane surface after a 24 hour contact with any medium normally used at 44.5 C incubation. Heavy imprinting of the grid system can also result in a network of "canal-like" indentations that frequently become filled with confluent growth.

The physical structure of the membrane filter material should be such as to provide an optimum retention of bacteria on the surface with little migration to areas within the pore matrix. When surface penetration occurs, growth may be limited in development during the colony counting procedure.

Chemical composition of membrane filters has largely been limited to polymerized cellulose esters since membrane filter technology initially developed in this direction. Conventional media designed for selective recovery of bacterial indicator groups or pathogens using agar pour plates, streak plates or broth cultures had to be redesigned to compensate for the physical-chemical properties characteristic of nitro-cellulose materials (29, 40, 41). For example, the selective adsorption of dyes excluded the use of acid to neutral dyes as indicator systems and necessitated the use of increased amounts of brilliant green as a suppressive agent in Kaufmann's Brillian Green agar to obtain the desired suppression of some of the unwanted bacterial population. Similarly, various nutrients such as tryptone, polypeptone and proteose peptone No. 3 were found to be superior in membrane filter media than in the same media used originally with peptone in their formulation. The result has been the creation of a family of media designed specifically for use with nitro-cellulose membrane filter products. With these experiences in mind, manufacturers should be careful about revising the Goetz membrane filter process. Changes involve the risk that recommended media may suddenly become less sensitive or less selective. Some compounds introduced to the membrane filter may improve flexibility, flow rate or stabilize porosity. However, these substances should not become a source of fermentable carbohydrates that cause false colony differentiation, create pH shifts in the indicator systems, are selectively toxic for specific organisms, or adversely depress the selective action of differential media by providing the bacteria with a highly nutritive organic compound. In essence, membrane filters should remain inert to bacterial reaction, and unchanged in those physical-chemical characteristics that effect media selectivity and sensitivity.

Sterilization of the membrane filter is essential to all applications involving filtration of liquids for bacterial removal or for use in bacterial cultivation. Prior to the development of the Goetz membrane filter process, membrane filters were sterilized in the laboratory by gentle boiling in distilled water for 20 minutes and repeating the procedure a second time with fresh distilled water (15-29). This procedure served the double purpose of sterilizing the membrane and of extracting any residual toxic substances. In retrospect, the continued use of this leaching and sterilization procedure would have avoided many of the variations in membrane filter performance now evident. However, the procedure does take more time to execute and is a recognized inconvenience in busy laboratories examining 50 to 200 samples per day.

Goetz proposed the use of ethylene oxide (0.5 ml per liter volume) for 3 to 4 hours at room temperature in a dessicator followed by air flushing for several hours to remove the sterilizing gas residual. This sterilization procedure is very effective but requires a thorough flushing, preferably in a vacuum system at an elevated temperature for several hours, with the procedure being repeated on two or three succeeding days for complete removal of gas entrapped in pockets in the membrane matrix. As a safety precaution relative to the explosive nature of pure ethylene oxide in air at certain concentrations, it is more desirable to use a mixture of ethylene oxide and carbon dioxide to decreased explosive and flammable properties.

With improvements in membrane filter technology. subsequent research demonstrated that membrane filters (packed with absorbent pads at top and bottom of a stack of filters wrapped in Kraft paper) could be sterilized prior to use by autoclaving at 121 C for 10 minutes. Immediately following the time-temperature exposure, the autoclave should be rapidly exhausted to atmospheric pressure and the membranes promptly removed to minimize total heat exposure. Excessive exposure to sterilization temperatures can cause membranes to become brittle and distorted. This problem is also aggravated by sterilization of membrane filter stocks held in storage for periods beyond 18 months.

The introduction of prepackaged and presterilized membrane filters in resealable envelopes by several manufacturers was considered a desirable convenience by the laboratory and immediately accepted. Now we have evidence from a recent comparative study of these presterilized membrane filters that there are significant increases in bacterial recovery rates for steam sterilized membrane filters compared to ethylene oxide presterilized membranes (37). As a result, one manufacturer that previously used ethylene oxide sterilization is now reported to be using gamma radiation for sterilizing membranes packaged in single service envelopes while another manufacturer has switched to steam sterilizing their packs of 10 menbrane filters. For laboratories that currently have supplies of ethylene oxide sterilized membranes it may be desirable to submit them to steam sterilization (121 C for 10 minutes with rapid steam exhaust) to further flush out latent toxicities. These membranes should then be compared with other membranes from the same lot of ethylene oxide treated membranes in a pure culture recovery experiment. Possibly some residual toxic effect may still persist either from entrapped ethylene oxide or its reaction products.

Despite manufacturing claims to the contrary, nitro-cellulose membrane filters do undergo some change in their physical characteristics upon storage in the laboratory for periods beyond 18 months. Upon aging, membrane filters may lose their flexibility and break apart at pressure points created during manipulation. During filtration, surface warping often occurs making a complete contact with the medium substrate impossible. The solution to this problem is not to stock pile membrane filter supplies beyond what is estimated to be needed for a 12 month period.

Other Variabilities in the Membrane Filter Test

The quality of the membrane filter is not the sole source of unreliable performance. Bacteria retained on the MF surface may receive nutrients from a broth saturated absorbent pad or from an agar based medium. When a liquid culture medium is preferred, the absorbent pad substrate material must be of high quality paper fibers, uniformly absorbent and free of sulfites, acids, or other substances that could inhibit bacterial growth. Recent quality control testing of absorbent pads supplied with membrane filters of various manufacturers, has demonstrated a significant reduction in colony counts and colony size associated with use of the absorbent pad substrate in comparison to the same medium prepared in a 1.5 percent agar base (35). Until the absorbent paper quality improves, it will be necessary for the laboratory to remove residual toxic materials, such as bleaching agents, by presoaking pads in distilled water held at 121 C for 15 minutes in the autoclave, decanting the rinse water, and repackaging pads in large petri dishes for sterilization at 121 C for 15 minutes, using a rapid exhaust to quick dry the pads (22).

The alternate approach is to prepare all MF broths with the addition of 1.5 percent agar. However, it should be noted that these agar preparations must be carefully added to culture dishes so as to create a smooth, moist surface, free of pock marks caused by foam and rapid mixing of air bubbles in the liquid agar preparation.

Media manufacturers have also contributed significantly to the membrane filter dilemma through variations in media quality (42). Formulations of media currently available and recommended in various reference sources contain a variety of peptones, bile salts and dyes which are not chemically pure compounds and thus subject to variations in composition and performance. As a result, medium sensitivity and selectivity will vary unless manufacturers maintain an adequate quality control program to insure that these products meet the requirements for their intended use. Although a quality check is made of these commercial products (43). it appears to be inadequate. Poor quality total coliform sheen development and significant reductions in coliform recovery on M-Endo medium have been observed by several laboratories in recent years. Apparently the use of poor grades of basic fuchsin and inadequate dye-sulfite balance in the medium are responsible. Basic fuchsin may differ in dye content, both from lot to lot and from manufacturer to manufacturer, making it essential to standardize the fuchsin-sulfite proportion used each time a new lot of dye is employed (22). Variations in intensity of the blue color of fecal coliform colonies on M-FC medium may be caused by residual acidity in absorbent pads or membrane filters and also from unsatisfactory lots of aniline blue used in the commercial preparation of this medium. The intensity and structure of bile salt crystals that precipitate on fecal coliform colonies relates to the type of bile salts complex incorporated in the medium. Formulations of commercial media containing sodium azide (M-Enterococcus, KF and PSE agars) have an approximate shelf life of two years after production, because of the deleterious effects created by the slow decomposition of the azide compound. For these reasons it is desirable for the laboratory to establish a quality control analysis on each new lot of medium purchased, comparing it with a lot of the same medium known to be satisfactory in terms of differential qualities and sensitivity.

No discussion on the variability of any laboratory technique can be complete without a recognition of the human element. Reliable laboratory performance by every technician in the determination of bacterial quality of water requires the continued application of knowledge, skill and judgment. Only through a uniform application of careful technic and rigid adherences to details will the procedures yield the maximum benefits of reliability and accuracy (32). Deviations in laboratory procedures occur as a result of many factors including attempted shortcuts, ignorance of technical procedures, inexperience in new methods, equipment failures, inadequate facilities, technical carelessness, shifts of competent personnel to other laboratory assignments, and lack of interest in the phase of public health bacteriology (28). These sources of variability in the membrane filter procedure can be held to a minimum through a vigorously pursued certification program at both the Federal and State levels. This program can best be achieved through training in proper methodology, supported by periodic laboratory evaluations and a bacteriological reference sample protocol to test laboratory proficiency and to reaffirm the continued production of reliable data (44).

Special Membrane Filter Application Considerations

The unique properties of the membrane filter and the compactness of basic apparatus stimulated the development of several devices that appear to have special potential for field use application. One of these devices, the field monitor, serves initially as the filtration chamber and then as the culture package upon injection of concentrated modified media into a pad below the filter.The unit is then ready for incubation and subsequent colony counting. Several independent evaluations of the field monitoring concept for total coliform recovery from polluted water indicate that only 70 percent recovery of the known bacterial density is being obtained. The remaining organism loss occurs from : a) some bacterial bypass around the filtration area to the pad below the membrane or direct to discharge through the bottom port; and b) failure of some debilitated cells to grow on the membrane and medium. In an attempt to seal off the by-pass loss, the manufacturer has added a hydrophobic substance to the outer periphery of the filter. Unfortunately, one lot tested in our laboratory had a much reduced effective filtering area due to the non-wetting agent. Inclusion of a consistent amount of normal strength medium is dependent upon displacement of the water entrapped in the pad with 1.3 times normal strength ampouled medium filtered through the field monitor following water sample filtration. Vapor blockage and uneven flow through will result in uncertain medium concentrations in the pad substrate, ultimately affecting bacterial growth. Ampouled media has a limited shelf life that must be recognized by the laboratory; 6 months for M-FC and 18 months for M-Endo when stored in the dark, preferably at refrigerated temperatures.

The bacteriological "dip stick" appears to offer the ultimate yet achieved in test simplicity at some sacrifice in flexibility. This device consists of a sterile rectangular shaped membrane filter positioned above a medium impregnated pad, both being secured to a plastic frame which is inserted into a mating plastic case. The basic principle of operation is the controlled absorption of one ml of sample through the membrane to the medium impregnated pad of critical thickness when the "dip stick" is held in a water sample for approximately 30 seconds. The small volume of sample makes the "dip stick" self limiting for total coliform analysis in potable water because the test base-line is established as "less than one coliform per 100 ml." Preliminary evaluation of the "dip stick" for use as a standard plate count measurement in potable water compared to the Standard Methods procedure shows the method to result in significantly lower bacterial counts, possibly because of the toxicity inherent in the gray-black membrane filter and to the inadequately enriched medium. The fecal coliform "dip stick" appears to offer the field investigator a convenient preliminary screening tool for water pollution surveys. However, before this procedure can be accepted as producing definitive data for stream standards and effluent qualities, it must be evaluated on a variety of waters including acid mine drainage, highly nutritive paper mill wastes and chlorinated effluents. The critical unknown factors involve the effect of source water chemistry on indicator bacterial survival and suppression of false positive reactions from non-indicator organisms. In this respect, a critical need exists to determine the adequacy of the "dip stick" fecal coliform procedure for monitoring chlorinated sewage. Consistent reliability of the bacteriological "dip stick" has yet to be determined and will relate to basic qualities of the membrane filter, absorbent pad and media stability during storage.

Special qualities of the membrane filter must be recognized by the researcher involved in radioactive detection of bacterial indicators. An adverse effect of membrane filters on bacterial release of carbon labeled CO_2 from radioactive tagged sodium formate was observed by Levin et al. (45). Filtered cultures of bacteria invariably released much less radioactive CO_2 per cell than did cultures that were not filtered. Either toxic material from the membrane filter inhibits metabolic activity of the bacteria and thus, the release of radioactive CO_2 or mechanical rupture of some of the bacterial cells by vacuum filtration effectively reduces $^{14}CO_2$ release. Studies on the problem suggest that although there may be some mechanical damage to cells by impaction, the mere presence of the membrane filter in the growth medium along with unfiltered cells was enough to reduce the $^{14}CO_2$ release. The problem needs more research investigation so as to control this factor in radioactive carbon measurement in a rapid test for bacterial indicators in water.

Membrane filters can be used as one of the adsorbents for the concentration of virus from water (46, 47). The clogging effects of turbidity can be partially circumvented by use of membrane filters of 293 mm diameter. However, sterilization of this size membrane by autoclaving may result in increased brittleness, making UV sterilization more desirable. The choice of membrane filter material is critical, with nitro-cellulose membrane filters having a high adsorption affinity for virus particles at a low pH (in the absence of interferring substances) even to pore sizes 285 times the virus diameter (48, 49). By contrast, cellulose triacetate filters absorb few virus particles, even at pore sizes no larger than three times the virus diameter.

Some laboratories filter sterilize tissue culture media for cell line maintenance. This practice may introduce a toxic contaminant to the medium from detergents incorporated in the filter to promote filtration efficiency and sterilization by autoclaving (50). Therefore, it may be advisable to flush all membrane filters in hot distilled water followed by an ice cold saline rinse before use.

The recent development of a dialysis chamber with membrane filter side walls offers an excellent opportunity to study bacterial survival in a variety of natural and artificial water environments (51). Membrane filters used for this purpose must be sturdy enough to withstand the buffering effects of water currents and for this reason tear resistant micro-web membranes with a nylon backing are recommended. Substitute membranes with a reinforced backing must also be free of biodegradable materials that will encourage the development of microbial films over their surface, restricting the in-flow of water and solutes to interact with the bacterial suspension. Finally, it is of critical importance that these membrane filters be non-selective in their passage of solutes which might alter the chemistry of the water under investigation and thus effect the bacterial survival patterns produced.

SUMMARY

There can be no doubt that the membrane filter dilemma is real and needs urgent resolution. Membrane filter and media manufacturers must heed the outcries from anguished microbiologists and reevaluate their product and quality control programs. If not, these corporations risk the loss of their multimillion dollar market through a growing wave of no-confidence in any membrane filter procedures with subsequent abandonment of this heretofore approved laboratory tool in water microbiology. This indeed would be a tragic turn of events that need not happen.

The laboratory staff should not be influenced by advertising claims of product excellence which we pay for but may not be getting. Possibly 15 percent of the laboratory activity should be apportioned to a quality control program on membrane filter and media lots and a variety of routine quality control procedures. The researcher is also cautioned to include adequate controls in all experiments involving membrane filters so that variables introduced from this source will not adversely affect the data interpretation.

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QUALITY CONTROL OF MEMBRANE FILTER MEDIA

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ABSTRACT

The manufacturers of dehydrated membrane filter media provide quality control, lot-to-lot uniformity and relative stability of their products. The quality control procedures test the media components and the dehydrated products for suitable physical and chemical characteristics, microbial contamination and growth support. In addition, the dehydrated products are subject to performance tests based on the use of the specific media. However, the users must maintain quality control after receiving the media. Quality control procedures in the laboratory include age of product, storage conditions, accurate weighing of dehydrated media, good quality of distilled water, clean utensils, complete mixing and solution, controlled heating, accurate pH determinations, approved supplements only, and appropriate checking with stabilized test cultures. Tests that the user should perform on the completed media include observation of appearance, pH, sterility, and membrane filter performance with selected test organisms.

INTRODUCTION

The development of membrane filter techniques for the isolation, enumeration and differentiation of microorganisms in water, sewage, milk, foods, air, solutions, specimens, etc., created a need for culture media especially suited for use with these techniques.

Kabler and Clark (5) reported that formulas of most media for conventional use must be modified before the best results are obtained with the membrane. This modification may be in the form of changed quantities of ingredients or the substitution of nutrients. These authors noted that in many instances there is no correlation between results obtained with an agar medium using conventional methods and the same formula without agar when used on a membrane.

This need for specialized media has resulted in the production by commercial dehydrated media manufacturers of a broad line of media for membrane filter techniques, which are denoted by a "M" prefix to the product name. Dehydrated membrane filter media offer the laboratory the same advantages as standard dehydrated media – relative stability, lot-to-lot uniformity and the quality control "built into" dehydrated products by commercial media manufacturers.

QUALITY CONTROL BY THE MANUFACTURERS

As with other dehydrated media, membrane filter media are subjected to quality control procedures at least twice before reaching the user. Initially, the raw materials incorporated into dehydrated media are tested before inclusion in various media formulas. Peptones are examined for physical appearance of the powder, pH, clarity, and color, and for microbial contamination. Peptones are also subjected to growth support tests in which a single peptone serves basically as the only nutrient. Agar is tested for gel strength, clarity by nephelometry, color by colorimetry, gelation and melting temperature, pH, solution time, and visual appearance. Only satisfactory ingredients are employed in the manufacture of the various media.

The second check is performed on the dehydrated product. Dehydrated media are subjected to the same types of tests described for ingredients, such as appearance, pH, clarity, and color. Performance tests with microbiological cultures are based on the end use of the medium. For selective media, it is necessary to demonstrate satisfactory growth of desired species and inhibition of undesired species, recognizing that a selective medium represents a compromise in that the selective agent may somewhat inhibit strains of desired species as well as undesired species. The performance of differential media is assessed by the use of appropriate species, including those producing positive and negative reactions.

For example, the BBL laboratory evaluates M-Coliform Broth, the BBL equivalent of M-Endo Broth, by determining that the broth colorimetric reading is satisfactory, and that the pH after heating and cooling to room temperature is 7.2 ± 0.2. Growth support tests include the use of strains of **Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium**, and **Shigella sonnei**. The medium is tested for the ability to support growth following straight inoculation of 10^{-1} dilutions of test organisms, and for the recovery and differentiation of E. coli in the standard procedure by adding one ml of a dilution containing 30 to 300 organisms to 50 ml of water, followed by filtration through a membrane filter subsequently incubated on a medium-soaked pad for 24 hours at 35 C. Growth must be satisfactory and reactions correct. **Streptococcus fecalis** is also included in the performance evaluation, and growth must be inhibited.

The use of stabilized, freeze-dried cultures permit results to be predictable and reproducible on standard or reference lots of media.

QUALITY CONTROL BY THE USER

The need for an internal quality control program in the microbiology laboratory has been documented in numerous papers and has been legislated for laboratories involved in the interstate practice of laboratory medicine through the Clinical Laboratories Improvement Act of 1967 (3). Abuses resulting in poor performance have not been uncommon, despite the quality control procedures performed by commercial manufacturers on dehydrated media and media supplements before their release and directions on labels and on product or methodology manuals for reconstitution and preparation of final media and their handling (1, 2, 6, 8).

Some of the factors that may contribute to the preparation and use of unsatisfactory finished media from dehydrated materials when little or no attention is paid to the quality of the completed media include the following (9):

1. Incorrect weighing of dry material, through human error or use of a faulty balance.
2. Use of dry material taken from previously opened bottles, which may have deteriorated from exposure to heat, moisture, oxidation, or other environmental factors. The quantities in which media are purchased should be regulated by the rate of usage. Ideally, bottles of dehydrated media, once opened, should be used within a few weeks. The purchase of media in 1/4 lb bottles or in sealed preweighed envelopes is encouraged.
3. Incorrect measurement of water and use of tap water or water from a malfunctioning still or deionizing resin column. Water should generally meet the requirements of the United States Pharmacopeia (USP) XVIII (10) for purified water or be of proven microbiological quality.
4. Use of unclean containers or glassware, especially those contaminated with detergent or other chemicals.
5. Incomplete mixing or incomplete solution resulting in failure to prepare a homogeneous medium. With agar media, this may even product stiff medium in some plates and soft medium in others.
6. Overheating occurring during preparation and sterilization, or resulting from holding too long in the molten state before dispensing into plates, tubes or bottles, can result in the loss of productivity through hydrolysis of agar, carmelization of carbohydrates, lowering of pH, increase in inhibitory action, loss of dye content in selective or differential media, and formation of precipitates.
7. Improper determination of pH, resulting in the addition of too much acid or alkali. The pH of a medium should be determined electrometrically; the electrodes should be in contact with the solidified agar medium, which may be removed from a plate or tube and placed in a beaker.
8. Improper addition or incorporation of unsatisfactory supplements or enrichments, or addition of supplements at the wrong temperature, possibly causing alteration of the supplements if the

temperature is too high, or gelation of media before proper mixing if too cold.

9. Failure of the laboratory to subject samples of finished media to quality control procedures with stabilized test cultures before the media are used. Regardless of the amount of quality control built into a product, users should be aware of strain variations which influence recovery and growth and of the purpose for which each medium was designed.

STORAGE OF MEDIA

Once prepared, special attention must be paid to proper storage. The selective and differential membrane filter media are more susceptible to deterioration than similar routine culture media. For this reason, we recommend that broth media, prepared in sealed tubes or bottles for later use, be stored in a refrigerator maintained at 2 to 8 C and used within a couple of weeks. It is generally recommended that M-Coliform Broth (M-Endo Broth) and M-FC Broth be used within four to five days (4, 7). Plates of agar media should be prepared and used promptly, or be stored wrapped in foil or plastic to limit water loss and used within two weeks. Containers of prepared membrane filter media must be protected from light.

RECOMMENDED QUALITY CONTROL CHECKS BY THE USER

Checks that the user can perform on the finished media include:

1. Appearance – if the medium is off-color, or there is a change in appearance during storage or signs of drying, contamination or deterioration, the medium should be discarded.

2. pH – measured electrometrically at room temperature. The pH should be within ± 0.2 of that stated on the label.

3. Sterility testing – by incubating representative samples, for two or more days. Such sample tubes or plates should be discarded and not used for later culture work.

4. Performance – as a minimal test, we recommend the straight inoculation of 10^{-1} dilutions of test organisms. As a test of the complete system, we recommend the filtration through a membrane filter of a water sample inoculated with a diluted suspension of an appropriate organism, followed by handling and incubation according to the procedure established for that medium. This test is a quality control check for the entire system.

If a problem is encountered, users are encouraged to contact the manufacturer. The lot number of the product and dates on which the medium was received and first opened should accompany the observations.

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DISCUSSION

Geldreich: Did you say that in the development of M-Endo media BBL uses a colormetric method to determine the proper amount of basic fuchsin?

Power: There is some color determination. I think it can vary with the lot of dye involved, and there would be a range.

Geldreich: The problem that I am concerned about is that many or all of us probably know that the actual dye contents of these products vary considerably. Dyes such as basic fuchsin, aniline blue, brillant green, etc. are not chemically defined materials. Sometimes the microbiologist is working with a trade brand of material which was originally intended for dyeing clothes and not for preparing media. Therefore, basic fuchsin, as an example, may have a dye content anywhere from 88% to 99%.

Power: That would be taken into consideration in the formulation of the actual dye.

Geldreich: This is our concern. Years ago we suggested that the manufacturers perform a biological dye titration when they made up M-Endo media. This titration is done by holding the amount of sodium sulfite constant and running each new lot of basic fuchsin against it. Each batch becomes an individual lot of media and is checked for recovery of the organisms with good sheen and no evidence of toxicity. I don't think the manufacturers do this. We often find that these products have poor sheen and sometimes poor recovery. I think it is because the sulfite-basic fuchsin is not in the proper proportion.

Lane: I am quite sure that BioQuest titrates the dye content in their MF media just as Difco does. You are absolutely right that there is variation in the dyes, and one of the worst problems has been with the aniline blue. Basic fuchsin has not been too bad. The only way that the dye content can be determined satisfactorily is by performance test. If the test organisms (coliforms in this case) don't yield typical colonial morphology or color, the dye content is off. The manufacturers of dyes seem to have lost the control that they had years ago. It is very difficult to get a satisfactory batch of basic fushion, aniline blue or brilliant green. It is up to the manufacturer to standardize the media so that the morphology and total counts are satisfactory. We do titrate.

Power: We do have the standards. If you experience a problem or if you see a variation, I wish you would let us know.

Seidenberg: You made a comment that has me in a dilemma; you said the M-FC and the M-Endo agar plates can be kept for 4 to 5 days.

Power: Yes.

Seidenberg: We in EPA are very careful to discard M-Endo plates after 48 hours, because we have found color changes.

Power: I notice that our manual and APHA manuals allow 4 to 5 days.

Seidenberg: I believe that **Standard Methods for the Examination of Water and Wastewater** states 48 hours.

Power: I believe that the holding times in the Standard Methods for Water and Dairy Products may differ by one day or so.

Geldreich: Standard Methods allows the worker to hold M-Endo and M-FC plates for use during that work week. However, I think some of the laboratories have found that these media are light sensitive and if they don't store them in the dark there is a real problem. One could cover half a plate, leave it on a laboratory bench about two hours, take that cover away and find two shades of media have resulted.

Power: I think it is best to use the media on the day of preparation.

Geldreich: If possible, that is great. Many workers ask how long they can keep the media. The current Standard Methods recommends a work week. This recommendation may be changed in the future.

Brodsky: I question the use of pure cultures as the only quality control that you use on your media. We are not accustomed to receiving pure cultures in a water sample. The relationship between the various organisms in their natural environment is negated by the use of pure cultures only. Perhaps pure cultures are used just because they are expedient.

Power: The use of the pure culture gives us a base for the comparison from lot to lot. Perhaps out of this meeting you will come up with some improved system as you indicated. Out of all the possibilities one might select, I frankly don't know which is most practical. We feel that we have organisms on which we have a history and back over the years we have used these cultures to test many lots. We are trying to give the user a product now that relates to previous lots, to keep them as constant as possible. One of the purposes of the meeting is to talk about the membranes, pads and media and to come up with final specifications.

Lane: The manufacturers of media must have a base line and the only base line we can presently use is pure cultures of **Aerogenes**, **E. coli**, or **Salmonella.** However, the final assay, the final approval, and this again is true for BioQuest as well as Difco, is the use of a natural specimen. One can titrate a selective medium using a stock culture, and can get certain results, such as selectivity. We recognize that when we use natural specimens such as natural water, polluted water, sewage and stool specimens, we get entirely different results. The statement that the manufacturer does not take into consideration the different results that could be obtained with natural specimens may not be correct.

There is one point I would like to raise; this is a problem that we encountered recently. We are taking part in the revision of **Standard Methods for the Microbiological Examination of Dairy Products.** We were using a buffered diluent as in water microbiology. We found that different lots of phosphate gave different total count results. Not only the different lots of phosphates but the time that organisms survived in these different lots varied. The organism will survive for an hour in one lot of phosphate but in two hours the count goes down. The count for another lot might be satisfactory at zero time but the count may go down after being held for 15 minutes. We also encountered the problem of the variation in distilled water when preparing a buffer from a good lot of phosphate. This is a problem that nobody has mentioned, and I think that you must consider this.

Bordner: In water analyses we are aware of the potential toxicity of phosphate buffers and the importance of good quality distilled water. **Standard Methods** allows an alternate dilution/rinse water, 0.1% peptone. The next edition will recommend the addition of magnesium sulfate to the phosphate buffer as an added protective against toxicity. There is also a test for the suitability of distilled water provided in **Standard Methods.**

I have a two-fold question. You have stated, Aaron, that you periodically use natural samples. Could you give us any estimate on your sampling frequency or upon what percentage of the media sampling per lot is based. Secondly, do you gentlemen have any suggestions on how we as water microbiologists could work more closely with the media manufacturers as I know you do with other groups. Could it be done with ASTM or other organizations and could it be a continuing relationship?

Power: That is why we are here, partly to tell you our story and mostly to listen to what you have to say, because obviously you have problems. If we can be of help, we certainly intend to cooperate. Until this week, I haven't been totally aware of all the problems involved and I certainly am willing to take back to my company anything that I hear. We are very willing to work with you and try to come to some solutions.

Lane: We check every lot of the M-Endo broth MF; for example, every lot is checked with river water. The other membrane media are not checked as frequently. We spot check them; perhaps 1 out of 4 or 5 lots. The basic media are checked every time we make a new batch. Concerning other media, for example, media for isolation of **Neisseria gonorrhea**, all media are assayed with clinical specimens. All media for isolation of **Salmonella** and **Shigella** are checked with stool specimens which are seeded with **Salmonella** and **Shigella**. Unfortunately you can't obtain natural specimens containing these pathogens so we seed them with different concentrations. I am sure that we do the same with the medium in which you are primarily interested, the M-Endo broth MF or the M coliform medium. We do check them with river water every time a batch is prepared.

Brezenski: I want to get back to the statement concerning the variation in dye content, because I feel this is very important. Every time a laboratory gets poor results there is a tendency to say there was a problem with the medium. The microbiologist talks to the manufacturer and he says, "well we have a problem with the raw ingredients which we don't have that much control over." I am wondering whether the manufacturer, for example Difco or BioQuest, specifies the amount of the active dye ingredient that is supposed to be in aniline blue and basic fuchsin and what the percentage of inactive ingredients should be. These specifications could then be established to conform to the quality control. For example, in the FITC dye used in fluorescence antibody CDC sets up a specification; they must have 80 to 90 percent active dye and only about 10 percent inactive ingredients. If the dye doesn't meet their specifications, they do not buy it. I am wondering whether Difco or BBL have specifications like this; because if we don't, this is where we should start.

Power: We do and on that particular product our specification exceeds the CDC specification.

Brezenski: The statement was made that variation is a problem; where does the variation occur?

Lane: There is variation in natural dye content. When you are comparing a fluorescent dye with an aniline blue and a basic fuchsin you are comparing almost a pure chemical with a mixture. The only way that you can test and approve or reject a batch of basic fuchsin or a batch of aniline blue is by performance. You cannot set specific criteria for actual dye content. You will have a variation, you will have a range of actual dye content, but the remaining components of a batch of basic fuchsin are really not specific materials. They are not even identified, so specifications can't be set. The only specification that can be set for any culture medium is its performance. Does the medium do what it was designed to do? Does this coliform medium, the MF medium that you are using to isolate the maximum number of coliforms present in that sample, yield colonies which are characteristic? This is what you are after.

As you pointed out, there are many variables in the dyes. Let me use peptones as another example. The peptone is not a specific substance. A peptone is either an enzymatic digest or acid hydrolysate of meat and casein. The method of production may be the same and the control of production for dyes as well as peptones may be the same; but the final criterion of a good peptone or a good dye is how does it perform in the medium.

STATISTICAL INTERPRETATION OF MEMBRANE FILTER BACTERIA COUNTS

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ABSTRACT

Statistical design and interpretation of experiments for enumerating bacteria by membrane filters are discussed. To increase precision of mean counts, large numbers of replicates can be used. In fecal coliform tests, large numbers of replicates can be achieved using a water sample stabilized by dilution in phosphate buffered peptone. Another way of increasing precision is to reduce the scatter among replicates. However, the random fluctuations of bacterial density, even in a well mixed sample, place a lower limit on scatter. This limit is predictable as a theoretical minimum standard deviation, which is useful as a yardstick in comparing with experimentally determined standard deviations. Experimental designs should provide for randomization of procedural variables so that unexpected variables do not bias results.

INTRODUCTION

Comparing different methods for enumerating bacteria is a familiar endeavor in microbiology laboratories. These evaluations include comparing MF techniques with other standards such as pour and streak plates, comparing variations in procedure, comparing membranes of different manufacture, and evaluating new media in comparison to available ones. In addition, in production and quality control operations, membrane manufacturers are involved continuously in evaluating improved membrane manufacturing and processing methods in comparison to existing ones and in qualifying production lots of membranes against standards.

The present paper discusses experiments in which the same water source or culture is used to test different membranes, media or procedures. An example is to evaluate effects of sterilization on membrane filters. You may have had the sad experience, as we have on some occasions, of reviewing pages of data only to find that each reviewer reaches different conclusions from the study! We will address here the formidable problem of carrying out an experiment leading to conclusions upon which all concerned can agree.

The factors contributing to a successful experiment can be categorized as statistical factors, experimental design principles, and bacteriological considerations. Statistical factors include decisions on the number of replicates to be used, the analysis of the scatter in counts expressed by the standard deviation of replicate counts and calculations of confidence limits. Experimental design must provide for isolation of the differences under investigation from extraneous effects; random selection of samples to be tested is important here. The bacteriological considerations include choice of the water source or the culture to be used, establishment of a detailed procedure, and selection of a useful control method. This paper is concerned mainly with statistical and experimental design factors; some of the bacteriological considerations in fecal coliform tests will be discussed briefly.

Statistical Factors

The precision of a count determined by averaging n replicate plates can be expressed by **confidence limits**, which give a range in which the true mean will lie. For example, the "95% confidence limits" are given by ± 1.96 $\sigma/\sqrt{n}$, where $\surd$ is the standard deviation of the population of individual measurements. The experimental mean has a probability of 95% of being within ± 1.96 $\sigma/\sqrt{n}$ of the true mean. To increase precision it is necessary to reduce σ or to increase n. Increasing n requires an operating procedure which is identical for each replicate and a water sample which is stable for the life of the experiment. An example of sample stabilization is given next. The scatter in replicate counts, which is measured by σ, is considered afterwards.

Fecal coliform counts for sewage samples diluted three different ways are given in Figure 1. Each plate was prepared by spreading a 0.1 ml aliquot of diluted sample onto M-FC agar. The lower graph, representing sterile water and phosphate buffer diluents, shows that the sample is unstable even for periods as short as 15 minutes. Use of 0.1% buffered peptone (prepared by adding 1 g peptone to 1 liter of phosphate buffer) stabilizes the sample for a much longer period. "Example 1" of the buffered peptone data exhibits a stable count throughout the entire two hour test period. In "Example 2", however, the counts begin to fall outside the 95% confidence limits at a dilution age of 60 minutes. Even with a 60 minute limit, however, peptone stabilized samples can be used in running far more replicates than could be used with water or phosphate buffer dilutents.

As illustrated earlier, the confidence limits around an experimental mean depend not only on the number of replicates, but also on the standard deviation of individual measurements. An experimental value, s, of the standard deviation can be calculated from each set of replicates. However, there is a particular source of scatter in membrane filter counts which is **predictable**, and it is possible to generate a theoretical value of σ, against which experimental values can be compared. The predictable source of scatter is the fluctuation in density of bacteria throughout a water sample.

At best, bacteria in a carefully mixed suspension will be distributed randomly through the surrounding medium. At worst, they may be associated with particles, adhering in clumps, or concentrated at the walls of the container. If they are

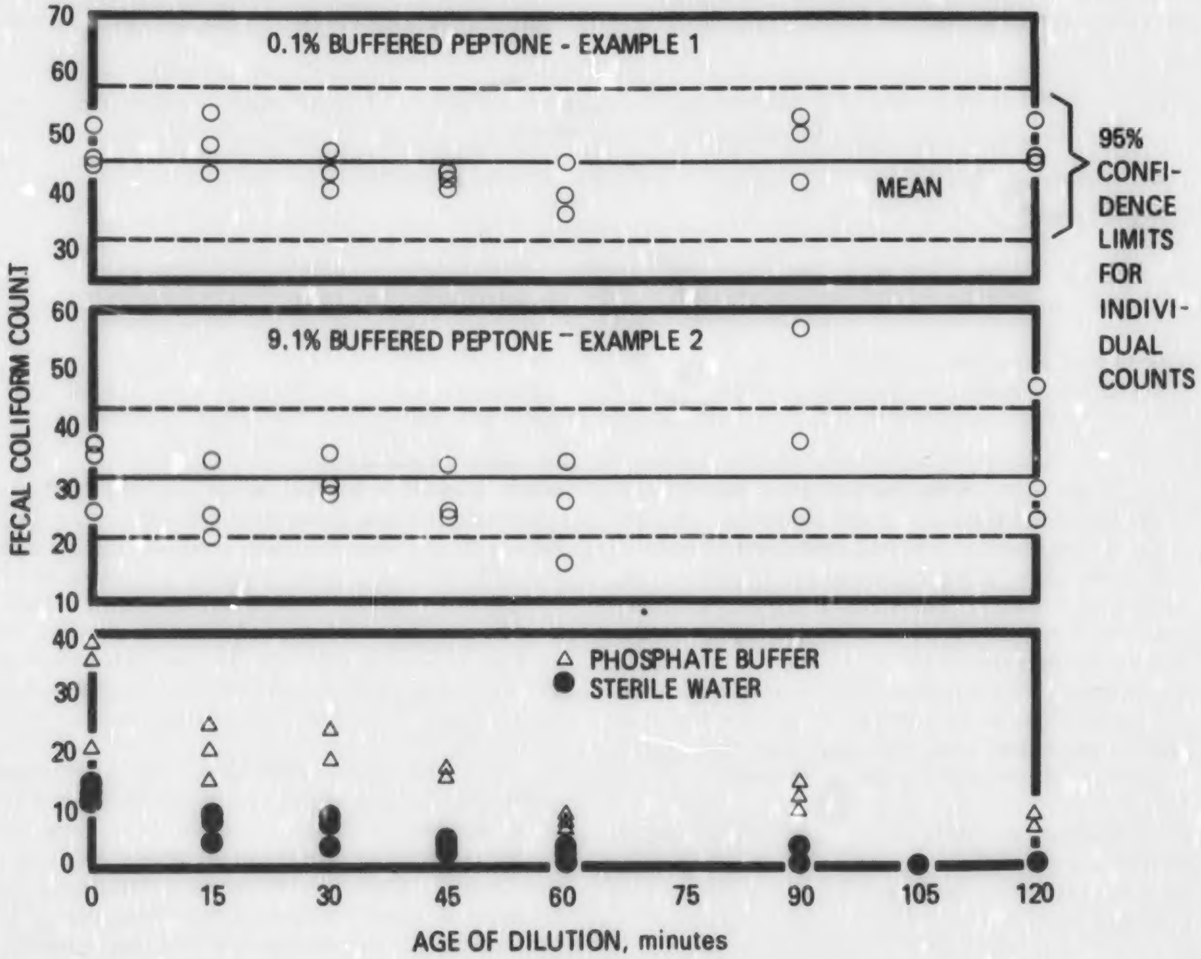

Figure 1. Effect of Diluent on Stability of Water Sample.

randomly distributed, samples of identical volume will not contain exactly the same number of bacteria.

The distribution of sample counts of particles taken from a random distribution in a suspending medium is called a Poisson distribution (2, 3). This distribution has a standard deviation equal to the square root of its mean. For the range of counts of usual interest in bacteriology, the Poisson distribution can be closely approximated by a normal distribution, which simplifies calculations considerably. Using this simplification, if the mean count per aliquot of sample is 100 bacteria, the standard deviation σ_R due to random distribution of bacteria through the liquid is $\sqrt{100}$ or 10. The 95% confidence limits on individual counts are ± 1.96 σ_R or ± 19.6. In other words, if the average count is 100, we can expect 95% of the measured counts to fall between 80 and 120, if the random distribution in the water sample is the only source of scatter in the data.

In summary, with the best case, random distribution of bacteria in the sample, we can expect a fairly large but predictable standard deviation. One example of using σ_R is given in the upper two graphs of Figure 1. The value of σ_R is the square root of the mean $\overline{X}$, found by averaging all the counts in the experiment. Then the predicted 95% confidence limits for individual counts are $\pm 1.96 \sqrt{\overline{X}}$. These limits are represented on the figure as dotted reference lines, showing when the experimental points are within acceptable limits.

Another example is to compare an experimental standard deviation, s, with the theoretical minimum standard deviation, σ_R. This will be done in a later section.

So far only one contribution to the scatter in an experiment has been considered. There are, however, other sources of error arising from variations in experimental technique. These can appear either as bias in the observed mean, $\overline{X}$, or as scatter in the data, an increase in s. In the following section, we will discuss how an experiment can be designed to prevent an unwanted bias from entering into the results.

Experimental Design

An example design is a study of the effect of ethylene oxide sterilization on mixed cellulose ester membranes. The purpose of the experiment is to isolate the effect of sterilization and hence it is necessary to eliminate effects of all other variables, known and unknown. The idea is to take a group of identical membranes, to sterilize half, and to compare these with the other half, which are not sterilized. The isolated variable is sterilization, but what about possible unknown variables: are the membranes really identical, is the experimenter's technique identical for each plate, is each water aliquot identical, is each plate incubated identically, is each counted the same way . . .? Although the bacteriologist exercises the strictest control over all of these factors, it is still possible for one of these or an entirely unknown variable to intrude into the experiment. To prevent unwanted bias, it is safest to **assume that unknown variables are present**, and to use randomization techniques to eliminate their effects.

Case	Number of Replicates, n	Experimental Mean, $\overline{X}$	Experimental Standard Deviation, s	Predicted Standard Deviation, σ_R
Streak Plates	18	76.2	14.6	8.7
Membranes, Unsterilized	18	119.4	17.3	10.9
Membranes, Ethylene Oxide Sterilized	18	123.9	21.8	11.2

For the sterilization study 36 membranes were chosen and coded for identification. Half were selected by random number techniques and were gas sterilized. Sterilization details are given in our paper, "Optimum Membrane Structures for Growth of Fecal Coliform Organisms" (1). Then each of the 36 membranes was selected in random order for testing. After incubation, plates were again randomized before counting.

The reason for all these randomizations was to "mix up" the effect of any undesired variables. Suppose, for example, that the technician who counted the plates became fatigued and biased the plates counted last towards lower values. If all the unsterilized membranes had been counted last, their lower counts would have been attributed erroneously to the isolated variable under study. Since, however, the plates were selected at random for counting, the (hypothetical) counting bias appeared scattered randomly throughout the results.

In summary, one can appreciate the value of random sampling by assuming the worst: namely, that in spite of your best efforts, **undesired variables are present.** By randomizing at each stage of the experiment, the undesired effects are scattered throughout the run so that they do not bias the effect of the isolated variable. To put this another way, the **signal** should be due to the variable under study and all other factors should appear only as **noise.**

Results of this sterilization study are given in Table 1. It is useful to compare experimental standard deviations with σ_R, as suggested earlier. Values from Table 1 are summarized below.

Here, the three experimental standard deviations exceed the values predicted for random dis-

TABLE 1. EFFECT OF ETHYLENE OXIDE STERILIZATION ON FECAL COLIFORM COUNT

Replicate Number	Streak Plate	Membrane, Unsterilized	Membrane, Ethylene Oxide Sterilized
1	58	127	152
2	76	128	135
3	68	129	139
4	74	85	100
5	89	115	93
6	120	117	121
7	65	143	109
8	74	142	87
9	80	135	143
10	88	114	90
11	56	129	124
12	81	102	118
13	89	135	144
14	66	125	134
15	65	112	120
16	75	126	165
17	71	102	127
18	77	84	130
Mean, $\bar{X}$	76.2	119.4	123.9
Experimental Standard Deviations,* σ	14.6	17.3	21.8

*Computed from $s^2 = \Sigma (X-\bar{X})^2 / (n-1)$

tribution of bacteria in the sample. This indicates that additional factors, such as the experimental variables discussed above, have contributed to s, and that randomizing at each stage of the experiment was a necessary precaution.

Some contrasting data on standard deviations are given in Table 2, which is a study of sterilization by three methods (1). Comparing s with σ_R, here, we see that two s-values agree well with σ_R, two are considerably lower than σ_R and one s is considerably higher than σ_R. To understand how an experimental s can be below the theoretical value, it should be mentioned that s has confidence limits and that these are quite wide unless very large numbers of replicates are used. The interested reader can compute the confidence limits on s using the X^2 distribution (2, 3).

The fact that the observed s values scatter on both sides of the predicted σ_R values suggests that the random distribution of bacteria in the sample was the only important source of scatter in these data, i.e., that scatter due to unwanted experimental variables was unimportant in this experiment. Hence, the confidence limits on the means shown in Table 2 use σ_R instead of s as the best estimate of the true σ. On the other hand, for Table 1 the experimental s values should be used as these contain the extra scatter due to unwanted experimental variables.

So far we have given several examples of the use of the predicted minimum standard deviation, σ_R. As a final example, the number of replicates needed in a particular experiment will be predicted. The example problem is to design an experiment so that a 10% difference in counts between two kinds of membranes can be detected, at a count level of approximately 100. If the mean ($\bar{X}$) from one type of membrane is 95 and the mean ($\bar{Y}$) from the other is 105, then we must make sure that the confidence limits on the difference ($\bar{Y}$-$\bar{X}$) are less than the difference itself (10 counts). The confidence limits on a difference involves the scatter in both values, and the squares of the two σ-values have to be combined to find the σ of the difference (2). That is,

$$\sigma^2{}_{Y\text{-}X} = \sigma^2{}_Y + \sigma^2{}_X$$

$$\sigma^2{}_{Y\text{-}X} = \sigma^2{}_Y + \sigma^2{}_X = \bar{Y} + \bar{X} = 200$$

The 95% confidence limits on the difference of means of n replicates are then $\pm 1.96\sqrt{200}/\sqrt{n}$ and an n of 8 or more is needed to give confidence limits below ± 10 counts. However, notice than an n of 8 will be sufficient only if the single significant source of scatter in the experiment is the random distribution of bacteria in the water sample.

Bacteriological Considerations

So far we have discussed statistical and experimental design factors using as examples two studies of sterilization of membrane filters. We consider here two of the underlying bacteriological considerations: Selection of the water source and the control method. Using again the example of sterilization, what water samples or cultures should be used to evaluate possible effects of sterilization on fecal coliform recovery on membranes?

If we regard the water source as an additional experimental variable, the concept of randomiza-

TABLE 2. EFFECT OF STERILIZATION METHOD ON FECAL COLIFORM COUNT

Case	n	$\bar{X}$	s	σ_R	95% Confidence Limits on $\bar{X}$*
Unsterilized Membranes	5	98.8	4.3	9.9	±8.7
Autoclaved Membranes	5	107.5	10.5	10.4	±9.1
Ethylene Oxide Sterilized Membranes	5	103.0	6.0	10.2	±8.9
Irradiated Membranes	5	94.3	9.4	9.7	±8.5
Streak Plates	10	82.0	14.2	9.1	±5.6

*Calculated from $\pm 1.96\,\sigma_R/\sqrt{n}$

tion is again useful. To randomize this variable it would be necessary to list all the water sources of importance for this type of test and to select at random a group for testing. Then at the end of the program results of all experiments would be averaged together to eliminate any bias due to water source effects. This kind of program is indeed a massive undertaking and requires the cooperation of several laboratories over a long period of time. We should thus regard the sterilization studies presented here, using two sewage sources, as only a beginning. A fine example of an extensive study involving three laboratories and a great variety of sources is given in "An Improved Membrane Filter Method for Fecal Coliform Analysis" later in this Symposium (4).

A second important bacteriological consideration is the selection of a control method. The spread plate was the non-membrane control used in the investigations of Table 1 and 2. The spread plate itself, however, is subject to a whole set of procedural details, one of which will be described here. Table 3 presents fecal coliform counts for 0.1 ml samples of diluted sewage on two thicknesses of M-FC agar. The same experiment was repeated on seven occasions using fresh sewage diluted in phosphate buffered peptone. Attempts to use agar thicker than 0.59 cm were not successfull due to the presence of spreaders. Looking at the overall means, there is clearly a significant increase in count with agar thickness.

The agar thickness effect illustrates that the spread plate control does not necessarily provide a complete measure of the number of viable bacteria. Although the spread plate does not provide an ultimate standard, we believe that it is important to include some non-membrane standard in membrane evaluations.

Table 3 also shows a possible water source effect. The first six runs show a statistically significant agar thickness effect, while the last one does not. If only the 12-27 run had been performed, we would have missed the effect altogether!

CONCLUSION

In conclusion, some of the factors entering into successful experiments evaluating membrane filters have been discussed. To provide narrow confidence limits on a mean count, it is useful to stabilize the water sample so that large numbers of replicates can be employed. The scatter in replicate counts, which also affects the confidence limits, cannot be reduced below a minimum which is characteristic of the random distribution of bacteria in the water sample. Procedural details also affect the counts, and it is important to randomize the order of procedures so that unwanted variables appear as scatter rather than as bias in the means. The sample source is also an important variable which should be randomized. Finally, non-membrane control methods are desirable in mem-

TABLE 3. EFFECT OF AGAR THICKNESS ON FECAL COLIFORM COUNTS ON SPREAD PLATES

Test Date	Fecal Coliform Count* On 15 ml Agar (0.25 cm thick)	Fecal Coliform Count* On 35 ml Agar (0.59 cm thick)	Size of Statistically Significant Difference**
11/12/74	40.8	49.7	7.6
11/14/74	30.2	46.8	7.0
11/15/74	68.7	94.3	10.2
11/20/74	24.8	46.7	9.3
11/21/74	48.8	69.8	8.7
11/30/74	60.8	82.6	9.6
12/27/74	27.3	27.7	5.9
Overall	43.0	59.7	3.1

* Each value is a mean of six replicates.

** At the 95% confidence level.

brane evaluation experiments, but these are subject to procedural variables, too, and do not necessarily provide a complete measure of the number of viable bacteria.

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QUESTIONS AND ANSWERS

Geldreich: In these studies on randomization you mentioned 18 membranes that you picked at random. Are you also talking about 18 random lots of membranes?

Sladek: No, 18 membranes.

Geldreich: How many lots are you talking about?

Sladek: This particular study was for the purpose of evaluating this one effect, ethylene oxide sterilization. This specific study was done with 36 membranes which were as closely replicated as we could choose.

Geldreich: Out of the same lot?

Sladek: Yes, they were identical.

Geldreich: You recognize that the variation of membrane filters from lot to lot is going to be another great variable that we are going to have to work with. Is this correct?

Sladek: Quite possibly.

Geldreich: We see it this way and I just wondered if you recognized this problem of lot to lot variation.

Sladek: I think, the best way that I could reply is to say, if you want to study sterilization, you should start with things that are identical. I think too often in the past people have found differences between membrane filters and may have attributed them to sterilization whereas in fact the differences were from other causes.

Geldreich: Part of this problem may be an interreaction between ethylene oxide and the membranes. Perhaps some of the products on the membrane may give a latent residual effect. Is there a problem?

Dawson: Mr. Geldreich has mentioned the possibility of residual ethylene oxide or hydrolysis products. Karl would you comment on residual ETO?

Saldek: I would be glad to. We have had analyzed a number of membranes that were sterilized by ethylene oxide. We had the membranes analyzed by an outside laboratory, the best one we could find. They never found any residual of ethylene oxide by chemical analysis. The limit of detection in that test was 4 parts per billion.

Sims: One problem that will be coming up over the next two days is: what is a lot? Is a lot the amount of raw materials you mixed, that you manufactured, or that you sterilized? One lot, number 500, may refer to each time the sterilizer was used; it can be each time a batch of raw materials were mixed; it can be each time the machine operated. This word 'lots' can refer to at least three different possibilities.

Sladek: I would like to comment on that. Its true that sometimes there are difficulties in defining a lot. However, once you have decided what a lot is, you get a lot of protection by doing random sampling and using a large number of replicates. For example, we have often sampled lots of membrane filters taking a sample of one hundred membranes drawn at random. This makes certain that if you didn't recognize some difference between lots when you were defining them, by random sampling you will pick up these differences in your quality control program.

Litsky: Would you give us your definition of a lot? What constitutes a lot? Would you like to give an answer?

Sims: I was actually asking what constitutes a lot for him. For me a lot is one day's machine operation. In our plant one solution is prepared per day. We sterilize about 4 times a day and we have to check each load after sterilization. I didn't know what they were calling a lot but the definition of a lot can be important when related to all of the data and quality control and experimental results.

Cotton: In our terminology we refer to a batch as that material produced at any one particular period of time during a day. For the lot numbering purposes we take a section of that batch and we may treat it in one fashion or another. We may autoclave pack it, or ethylene oxide pack it. That section of the day's batch which is produced and packaged in a particular way is considered and given a lot number.

EFFECT OF INJURY ON THE RECOVERY OF INDICATOR BACTERIA ON MEMBRANE FILTERS

Review of Author's Research

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ABSTRACT

Investigations have been undertaken which demonstrate that indicator bacteria, which are injured during aqueous suspension or by exposure to chlorine, may fail to form colonies on membrane filters incubated on selective media. Although dividing cells of **Escherichia coli** were recovered equally well on Trypticase soy agar and membrane filters incubated on M-FC medium at 44.5 C, the efficiency of cell recovery decreased with time of exposure to stress. **Streptococcus faecalis**, in aqueous suspension, was recovered on membrane filters incubated on KF agar and on M-Enterococcus agar, with an efficiency of less than 50%. Recovery of streptococci did not decrease with time of suspension.

These findings helped to explain results obtained by other workers and suggest the need to reduce the selectivity of membrane filter techniques against injured cells. Comparative studies of the recovery of decreasing, as well as growing, populations on rich and selective media ought to be included in evaluations of selective media.

INTRODUCTION

Most microbiologists have been concerned primarily with growth measurements of bacterial populations. The food and sanitary microbiologists are often required to estimate numbers of stressed indicator bacteria and pathogens. This paper reviews recent observations on the enumeration of declining bacterial populations in water, with special reference to the recovery of indicator bacteria on membrane filters.

Background

In recent years, an extensive amount of literature has appeared on the recovery of starved, heated, frozen and thawed indicator bacteria, leakage and degradation of cellular components, and the repair of cell injury. A limited amount of literature has appeared on the recovery of bacteria exposed to disinfecting agents. However, little effort has been made to evaluate the recovery of stressed indicator bacteria in aquatic environments.

In 1961, McCarthy, Delaney and Grasso reported that "weaker" coliforms failed to form colonies on membrane filters incubated on M-Endo broth. They found that pre-enrichment on lauryl tryptose broth, prior to incubation on LES M-Endo agar, improved recovery from surface water samples. Klein and Wu (3) recently demonstrated significantly higher recoveries of bacteria from stream waters on spread plates than on pour plates. These results indicate that a significant portion of the heterotrophic bacterial flora in the streams consisted of injured cells that were unable to tolerate the secondary stress of exposure to melted agar at 42, 45, and 50 C. Hoadley and Cheng (2) demonstrated injury of indicator bacteria suspended in a variety of aqueous suspending media. Injury prevented recovery on selective media.

In 1958, McKee, McLaughlin and Lesgourgues demonstrated reduced recoveries of coliforms from chlorinated settled sewage on membrane filters that were incubated on dehydrated scheduled nutrient pads containing an Endo-type medium. On the other hand, recoveries of coliforms from unchlorinated settled sewage on membrane filters, by most probable number tests, were in agreement. By applying the pre-enrichment procedure of McCarthy, Delaney and Grasso (7), Lin (5) was able to obtain agreement between most probable number values and MF counts of total and fecal coliforms in chlorinated secondary sewage on membrane filters incubated on M-Endo and M-FC medium. Braswell and Hoadley (1) described injury to **Escherichia coli** cells in chlorinated secondary sewage.

It is the purpose of the present review paper to describe briefly the recovery of injured and lag phase indicator bacteria on membrane filters employing standard selective media. Our results suggested the need to reduce the selectivity of membrane filter techniques against injured cells, and the need to understand cell injury better. It is encouraging that the recovery of injured bacteria is receiving so much attention at this symposium.

Hoadley and Cheng (2) examined the recovery, on membrane filters incubated on standard selective media, of **E. coli** and **Streptococcus faecalis** after suspension for varying periods of time in sterile stream water, double distilled water, phosphate buffer (3.125×10^{-4}M, pH 7.2), peptone water (0.01%), and tap water. **Escherichia coli** ATCC 11775 and **Strep. faecalis** ATCC 19433 were spread on plates of Trypticase soy agar (BBL) and incubated for 18 to 24 hr. at 37 C and 35 C, respectively. Suspensions of each organism were prepared in each of the sterile experimental suspending media and compared with McFarland barium sulfate standards to obtain an inoculum yielding a density of about 1000 organisms/ml after addition to the test suspension. One liter volumes of test suspensions were maintained at 20 C in water jacketed stirred flasks and samples were removed at intervals of up to 24 hours for counting.

Upon removal, samples were spread immediately in triplicate on Trypticase soy agar plates and were filtered in triplicate through membrane filters (type HA, Millipore Filter Corp.). Spread plates inoculated with **E. coli** were incubated at 37 C and were counted at 24 and 48 hours. Membrane filters carrying **E. coli** were incubated on pads saturated with M-FC broth (Difco) at 44.5 C for 22±2 hours. Spread plates inoculated with **Strep. faecalis** were incubated at 35 C and were counted at 48 hours. Membrane filters were incubated on KF broth (BBL) and M-Enterococcus agar (BBL) at 35 C and were also counted at 48 hours.

Counts of **E. coli** in double distilled water are presented in Figure 1. As a rule, counts on membrane filters initially resembled counts on the rich, non-selective control medium. But as the age of the suspension increased, so did the discrepancy between counts on the two media. Counts in double distilled water are presented because the discrepancy between recovery on the two media is greater than in phosphate buffer. Many tap waters may be highly toxic, even in the absence of residual chlorine. As a result, recovery on M-FC medium was very poor (Figure 2). Recoveries from peptone water and stream water (each of which contained between 545-550 mg/liter total organic carbon) were nearly identical. In each suspending medium, growth followed a lag period (Figure 3). During the lag period, the discrepancy between counts on selective and rich media increased until about 10 hours, after which cells recovered. Following recovery (18 hours), counts were nearly identical on the two media. Poor recovery of **E. coli** on a selective medium during the lag phase was observed also by Scheusner, Busta, and Speck (9).

The implications of these observations are clear. Cells of **E. coli** were injured during suspension in water, either as a result of leakage or degradation of cellular components. As a result, a substantial portion of the population failed to produce colonies on membrane filters incubated on selective media.

In contrast, although recoveries of **S. faecalis** were at all times low on both selective media, there was no evidence of progressive cell injury in most suspending media. Recoveries of **S. faecalis** suspended in distilled water and phosphate buffer were identical, and remained constant over a 24 hour period (Figure 4). However, recoveries on both KF medium and M-Enterococcus agar were about one half those on Trypticase soy agar. Recoveries from toxic tap waters on membrane filters were very much lower (Figure 5). **S. faecalis** grew both in stream water and peptone water following a short lag (Figure 6). Counts on each selective medium again were lower than those on the rich medium, but they reflected the behavior of the population as a whole. The consistent recoveries of **S. faecalis** from most aqueous suspensions on selective media is a desirable attribute in a recovery medium.

Recovery of E. coli from Chlorinated Sewage

Braswell and Hoadley (1) investigated the recovery on membrane filters of **E. coli** ATCC

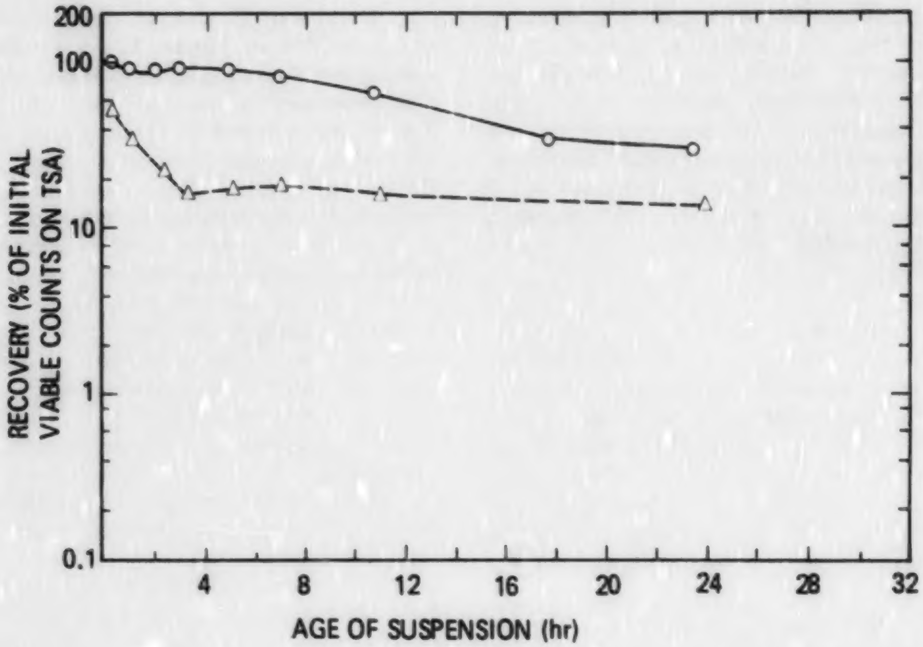

Figure 1. Recovery of E. Coli ATCC 11755 Suspended in Double Distilled Water on Trypticase Soy Agar (Circles) and on m-FC Medium (Triangles) (after Hoadley and Cheng, 1974).

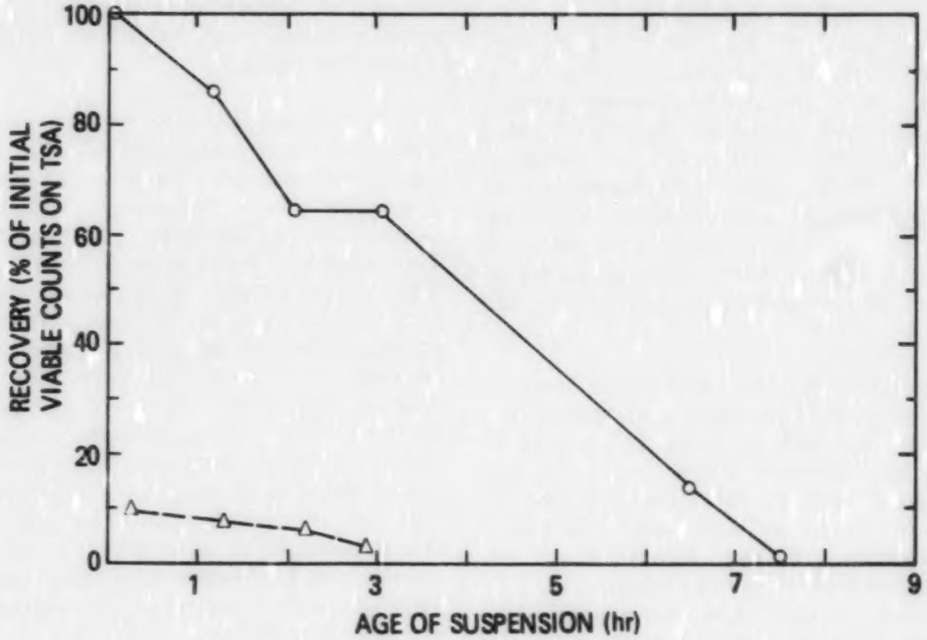

Figure 2. Recovery of E. Coli ATCC 11755 Suspended in Tap Water on Trypticase Soy Agar (Circles) and on m-FC Medium (Triangles) (after Hoadley and Cheng, 1974).

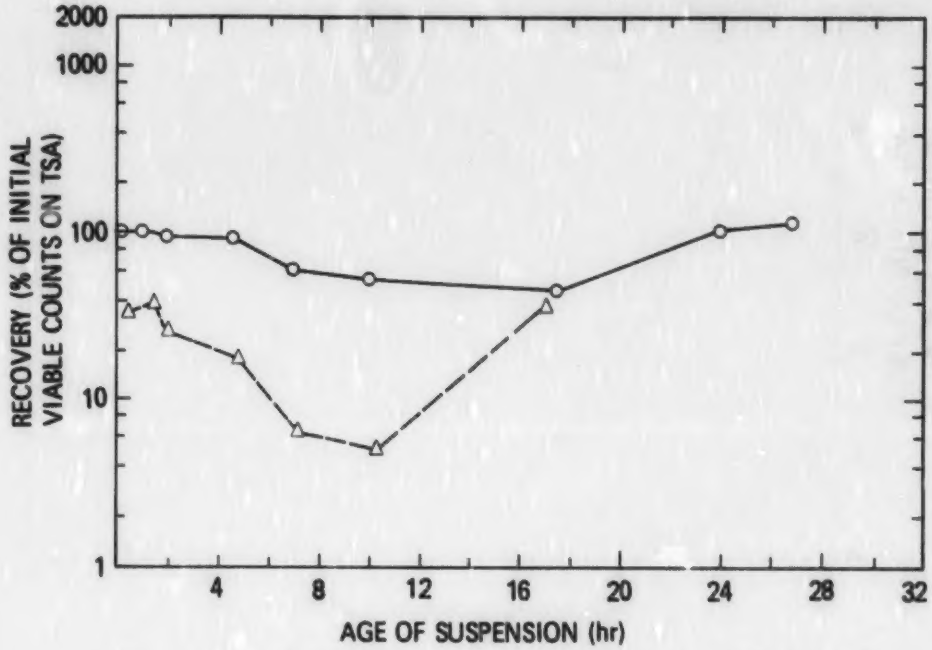

Figure 3. Recovery of E. Coli ATCC 11755 Suspended in Stream Water on Trypticase Soy Agar (Circles) and on m-FC Medium (Triangles) (after Hoadley and Cheng, 1974).

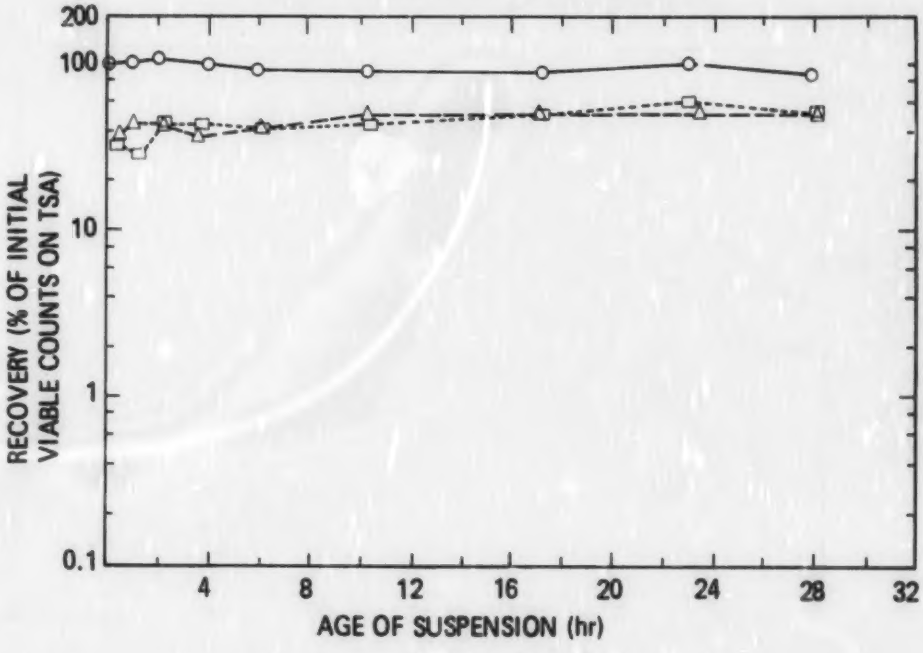

Figure 4. Recovery of Strep. Faecalis ATCC 19433 Suspended in Double Distilled Water on Trypticase Soy Agar (Circles), KF Medium (Squares), and m-Enterococcus Agar (Triangles) (after Hoadley and Cheng, 1974).

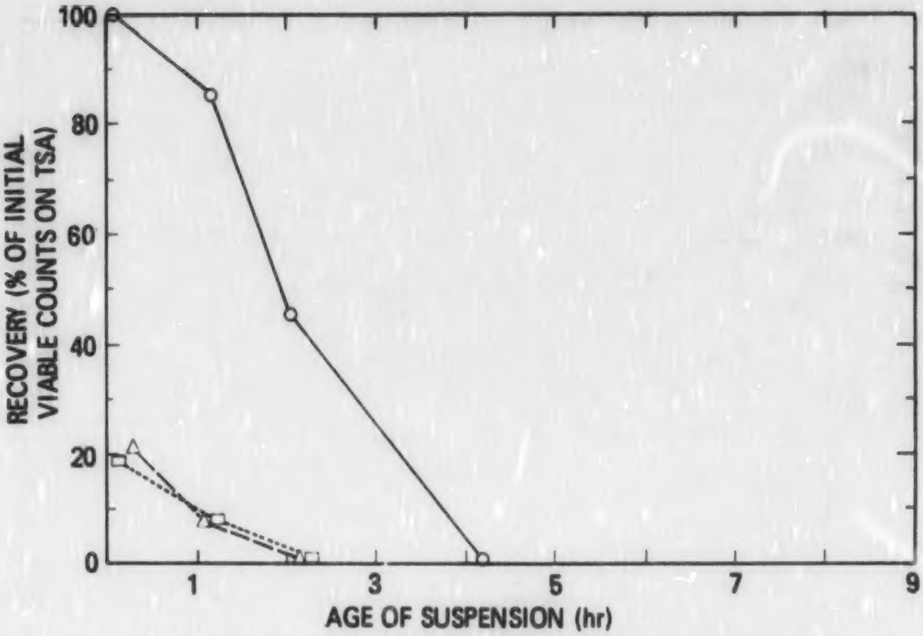

Figure 5. Recovery of Strep. Faecalis ATCC 19433 Suspended in Tap Water on Trypticase Soy Agar (Circles), KF Medium (Squares), and m-Enterococcus Agar (Triangles) (after Hoadley and Cheng, 1974).

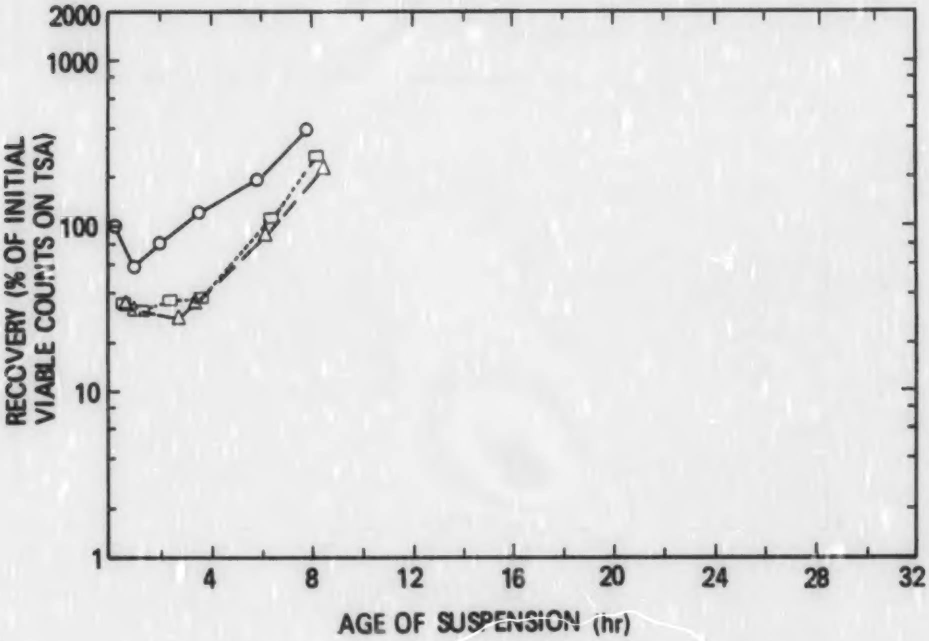

Figure 6. Recovery of Sterp. Faecalis ATCC 19433 Suspended in Stream Water on Trypticase Soy Agar (Circles), KF Medium (Squares), and m-Enterococcus Agar (Triangles) (after Hoadley and Cheng, 1974).

27622 suspended in secondary sewage and exposed to chlorine. A suspension of cells was prepared, as previously described, and was added to 500 ml of sterile sewage in a 1000 ml beaker to yield approximately 2×10^4 organisms/ml. A sodium hypochlorite solution was added to yield a dosage of 2 to 3 mg/liter and a total residual of 0.3 to 0.5 mg/liter after 30 minutes. Ten ml samples were removed after 1, 5, 10, 20, and 30 minute intervals, and were placed immediately into 10 ml of sterile sodium thiosulfate, after which appropriate dilutions were made. Diluted samples were spread in duplicate on Trypticase soy agar plates and filtered in triplicate through membrane filters (type HA, Millipore Filter Corp). Spread plates were incubated at 37 C for 24 hours. Membrane filters were placed on pads saturated with M-FC broth (Difco) and incubated at 44.5 C for 24 hours. In one experiment, filters were placed on Trypticase soy agar and on pads saturated with Trypticase soy broth.

Counts of E. coli in chlorinated secondary sewage are presented in Table 1. Counts on membrane filters generally were close to those on spread plates initially. With exposure of sewage to chlorine, however, recovery of E. coli on filters was decreased more rapidly than recovery on spread plates. In each of the experiments reported in Table 1, counts on the rich medium were still 1000 bacteria/ml after colonies failed to form on the selective medium. Scheusner et al (9) and Maxcy (6) reported that E. coli exposed to chlorine and other disinfectants did not form colonies on violet red bile agar. Poor recoveries of coliforms from chlorinated settled sewage on membrane filters reported by McKee, McLaughlin, and Lesgourgues (8), and enhanced recoveries following pre-enrichment reported by Lin (5), can be explained if it is understood that injury occurs during exposure to chlorine, thus preventing recovery on selective media.

A single experiment was undertaken to determine whether injured cells form colonies as readily on membrane filters as they do on agar surfaces. Spread plates were prepared as usual and samples were filtered through membrane filters, then incubated in triplicate on Tyrpticase soy agar, and pads saturated with Trypticase soy broth. All plates were incubated at 37 C for 24 hours. Results are presented in Table 2.

As in previous experiments, initial counts on membrane filters closely resembled those on spread

Table 1. Recovery of E. coli ATCC 27622 from chlorinated secondary sewage on Trypticase soy agar and on M-FC medium (after Braswell and Hoadley (1)).

Chlorine contact time (min)	Counts/ml			
	Expt 1[a]		Expt 2[b]	
	TSA[c]	M-FC	TSA	M-FC
1	16,000	15,400	40,000	14,000
5	1,600	17	26,200	1,400
10	1,000	0	8,600	460
20	10	0	1,000	0
30	6	0	100	0

[a] Temperature, 21 C; pH 7.0; chlorine dosage, 3 mg/liter; chlorine residual after 30 min, 0.75 mg/liter.

[b] Temperature, 21 C; pH 7.0; chlorine dosage, 2mg/liter; chlorine residual after 30 min, 0.35 mg/liter.

[c] TSA, Trypticase soy agar.

Table 2. Recovery of E. coli ATCC 27622 from chlorinated secondary sewage on Trypticase soy agar (Braswell and Hoadley, unpublished)[a]

Chlorine contact time (min)	Counts/ml		
		Membrane filter	
	Spread Plate	TSA	TSB[c]
1	33,670	33,570	30,600
5	31,630	28,070	22,370
10	20,700	14,630	4,800
20	500	45	1
30	30	2	---

[a] Temperature, 21 C; pH 6.9; chlorine dosage, 2 mg/liter; chlorine residual after 30 min, 0.4 mg/liter.

[b] TSA, Trypticase soy agar.

[c] TSB, Trypticase soy broth.

plates. However, after the initial sample, recoveries were lower on membrane filters, and again the discrepancy increased as time of exposure to chlorine increased. Furthermore, recoveries on membrane filters were poorer when incubated on pads saturated with Trypticase soy broth than when incubated on Trypticase soy agar.

Conclusions

It is clear from the above observations that we must pay more heed to the effects of injury on the recovery of indicator bacteria from water. While healthy, multiplying **E. coli** cells recover well on membrane filters incubated on selective media, cells exposed to stress may not form colonies on filters or selective media after suspension in surface waters, chlorination of wastes, or chlorination and passage of potable waters through distribution systems. Furthermore, it might be possible to devise media, superior to those employed, on which all viable cells are able to form colonies. The significance of the discrepancy between counts on rich and selective media is suggested by the observations of Speck and Cowman (11), that freeze-injured salmonellae may be as pathogenic as uninjured cells.

Evaluations of media and techniques should include examination of the recovery of stressed bacteria in pure culture. Cells of **Pseudomonas aeruginosa** stressed in fresh waters and estuarine waters were employed by Levin and Cabelli (4) to evaluate their M-PA procedure. Such evaluations might be applied profitably to existing, as well as newly developed procedures.

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Discussion

Brezenski: I would like to ask you one question with respect to the chlorination experiments. Do you have some of the other chemical characteristics of the secondary effluent that you use with respect to solid and other organic removals?

Hoadley: We don't have parameters like TOC, TSS, etc. These samples were just autoclaved and we used the same batches as much as possible.

Brezenski: We did some work several years ago in our laboratory comparing the recoveries of membrane filter and MPN systems from primary chlorinated effluents of sewage treatment plants. We experienced atrocious recoveries. The MF's , in every case, were many magnitudes lower. But, as we progressed through various stages of treatment, e.g., when we went up to secondary treatment and to effluents from good activated sludge plants, we found that the results were coming closer together. When we came to a tertiary plant, we found that we almost experienced comparable results. These results make me wonder if we are dealing solely with the recovery of injured cells. I agree with you 100% that we do have a problem of cells being stressed in the environment, and also in chlorinated waters. I also believe that we have to take into consideration the physical factors, for example, the level of suspended solids. It is quite evident from this data that if you do have a high level of suspended solids, the membrane filter is not going to give a good recovery. There is no question that McKee, in his early work in 1958, showed that chloro-organic complexes form on the membrane. And this does have a growth-inhibition effect on these colonies. I believe we are not only talking about a stressed system, we are also talking about a system of physical characteristics coming into play. I think the analyst, in this case, is going to have to determine when he sees the sample concerned, how he is going to treat it. It becomes difficult because now we have some subjectivity involved.

Hoadley: I agree. I think there are many factors that are going to influence our recoveries here. All I can do is demonstrate a phenomenon. There is an effect. Initially our recoveries are much the same. In all of this work we would grow the bacteria on plates, suspend the cells, adjust the turbidity and inoculate our samples. This took 7 minutes usually. Then we would run those things, initially the counts are about the same.

Alico: Two questions, the first is, how many replicates did you run and secondly what were the means of sterilization of the filters that you used?

Hoadley: We used the filters from the presterilized packages as they came. Usually tests were performed in triplicate. We have run various experiments on different strains from time to time, and you get variation from experiment to experiment; but the phenomenon is there.

Alico: What membranes were you using?

Hoadley: I don't think the brand is pertinent here, because we are looking at the phenomenon; but they were ethylene oxide-sterilized.

Lane: I would just like to point out here the importance of osmotic pressure and its effects on recovery of injured cells. For example, in clinical microbiology and blood cultures, if you take one broth, use it as is and add 10% sucrose to another bottle of the same broth, you will get a increased recovery of injured cells in the broth with the 10% sucrose. This has been demonstrated repeatedly, and I think you may have a similar situation here.

Hoadley: I think there are a lot of questions about the environmental conditions. I don't think we have good control of that in our experiments, and I'm ready to admit that. I imagine we were not doing things much differently than other people, so this is the way our techniques are applied.

Dawson: Just a comment. I think that osmotic pressure may play an important factor here but I think we also should look at the quality of the water we use for preparing dilutions in reagents. The gentleman from BBL indicated that he would like to see USP purified water used as reagent grade water. Purified water USP-18 is not all that good in the light of modern-day technology. Single distilled water can contain a high quantity of amines and we know that amines come over with a single distillation. There are numerous reports published in the literature that amines are toxic to both bacteria and to tissue culture cells and I wonder if we are seeing something here?

Hoadley: We would never use single distilled water at all. It becomes very clear in any of these comparisons that the better the quality of distilled water the better your recoveries are after suspension.

EFFECTS OF TEMPERATURE ON THE RECOVERY OF FECAL COLIFORMS

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ABSTRACT

The theoretical concept of cell and culture death resulting from increased temperature is important to an understanding of the inaccuracy of the fecal coliform procedure. Results of our studies on the differential effects of temperature on various types of membrane filters show that the type of filter is an important variable. Recoveries of known densities of pure cultures of E. coli varied from 10-70 percent from this variable alone. The importance of selection of the proper temperature for membrane evaluation cannot be stressed too strongly.

Results show that the interaction of the membrane with the cell, resulting in loss of cell viability, is temperature dependent and varies from brand to brand. A modified fecal coliform procedure has been developed and field tested. This new procedure lessens the detrimental effects of the membrane on recovery of the organism.

While this symposium is entitled, "The Recovery of Indicator Organisms Employing Membrane Filters", I sense that it is really an effort to re-establish the M-FC method, or a modification of that method, as recommended technique. I sincerely hope that this symposium and the discussions that follow will, in fact, provide investigators with a workable method.

It is no secret that there have been several reports over the past year and a half which have demonstrated poor results with the M-FC method under some conditions. Most of these reports have indicated that the fault lies in the quality of the membrane filter. As the author of one of those reports, I am certainly not going to deny that conclusion. I will modify it and say that the membrane quality gives **some** of the error and most of the variability.

I will limit my presentation to the M-FC method. The subject of my presentation is the effects of temperature on recovery, but in order to fully develop my topic I would like to discuss several aspects of our evaluations concerning the procedures that were used. I hope that I will not tread on the data of our other speakers.

In order to evaluate the problems with the M-FC procedure, one must first develop a method, based on sound reasoning, which restricts the variables with which one has to deal. I would like to begin by discussing some of these variables and describing our selection of a defined evaluation procedure. (Tab. 1).

The organism

Any organism selected as a test organism in evaluating this method must meet several criteria:

1) The organism must be a pure culture. This is not to deny the use of field trials; nor of their importance in the total evaluation of any analytical procedure. It is necessary, however, to first estab-

TABLE 1. VARIABLES

A. Organism
    1. Must be a pure culture.
    2. Must be easy to obtain by the investigator.
    3. Must be E. coli.
    4. Must not be selected for by the M-FC Procedure.

B. Medium

C. Filter membrane

D. Temperature

lish the validity of the test procedure using a known, pure culture of the organism which the test is supposed to quantitate. I stress this because some have proposed using mixed cultures. Mixed cultures have a way of changing their makeup depending upon how they are cultured.

2) The organism must be easy to obtain. Specifically it should be on deposit with a type culture collection and the strain number identified so that each investigator is not introducing his own variable. We have often found that we could not compare our results with other investigators because of the organism employed and the impossibility of isolating the same culture under the same conditions.

3) The organism must be **E. coli**. This point should be obvious. Notice, I did not say it should be IMVIC positive. True, **E. coli** is, but that test is not sufficient by itself to identify the organism. Such studies have never been done. It does differentiate the organism from **Enterobacter aerogenes** and so far that is the only use of the test. Nor did I mention other criteria such as lactose fermentation at 44.5 C, etc. There may be other organisms that meet that test. If it is **E. coli**, it meets all of the criteria. But if it meets all of the criteria, it may not be **E. coli**.

4) The organism must **not** be selected for by the M-FC Procedure. By using the M-FC Procedure to obtain the test organism, it is possible that one might obtain a mutant more resistant to the inhibitory effect. This would lead to erroneous results since the procedure itself is not designed to recover only those resistant types. We isolated such a colony and it gave 2.5 times the recovery as our stock culture.

In our work, we selected ATCC strain 11775 because it is the neo-type strain, and we felt that this organism best represented the organism for which coliform tests were originally developed. Cultures were grown in nutrient broth for 16 hours at 35 C.

The medium

The original investigators made the medium from scratch. Most people probably employ commercially available products. The question is, are all products equal in quality and do they vary from batch to batch. We had not had time to expand our studies into this area, but we feel that someone should. I am also concerned about the sources and quality of the rosolic acid employed. We have used both Difco and BBL media with no apparent difference. Our rosolic acid has always been from Difco. In order to remove a variable we are using Difco medium only, at the present time.

The filter membrane

We did some work comparing the different brands of membranes. The early work by Presswood and Brown, plus some of our own work comparing Gelman and Millipore filters, showed that while the brand of membrane has a decided effect on the recovery in the M-FC Procedure, it might not be the only effect. First we compared, Then we chose Millipore because it was the worst and we wanted to know why. Then we went to Gelman because it gave the best results and we would not have a second experimental variable.. And finally we decided to use none because we couldn't trust any of them. One gets that way when he opens a new package and finds nothing between the blue paper.

The temperature

Temperature in this method is very important. 44.5 C is not hard to obtain, but most incubators and waterbaths have a hard time maintaining ± 0.2 C throughout the chamber. For this reason we used a homemade circulating waterbath that we had been using for our enzyme work. We adjusted and checked it with a quartz thermometer at first, but we now use a thermometer which has been checked against an NBS standard thermometer. I have seen many people who simply reach in the drawer, pull out a thermometer and use it. Perhaps we need to stress standardization more than we do.

We have concentrated on this variable, temperature, and it is these results which I would like to discuss today.

What effect does temperature play in the recovery of coliforms by the M-FC method? Let's look at the results of several investigations comparing brands of filters. Presswood and Brown compared Millipore and Gelman filters. They found a mean loss on Millipore of 53 percent when compared with the Gelman filter. In their study, they concluded that temperature itself was not detrimental, but that the Millipore filter was. We disagree with those conclusions. Their organisms were isolated with the M-FC Procedure and their results were compared to cultures grown on M-FC agar pour plates at 35 C. Since the difference showed up only at the elevated temperature it would seem that temperature did have an effect. The question is, is the effect on the membrane, on the cells, or both. Recently Dutka, Jackson, and Bell reported on a comparison of six different filters. None of these filters gave better than 38 percent recovery at 44.5 C compared to a pour plate control at the same temperature.

When we compared Millipore with Gelman we found that the Millipore recovered only about 10 percent of the cells known to be in the sample. (Tab. 2) The Gelman filter, however, recovered about 55 percent. Our standard was the number of cells which grew on plate-count broth at 35 C. We feel this is the only reliable criteron for knowing how many viable cells there are in the sample.

TABLE 2. RELATIVE ERROR IN THE FECAL-COLIFORM METHOD AS A FUNCTION OF THE BRAND OF MEMBRANE FILTER EMPLOYED.

		No. of E. coli cells per 100 ml		
Experiment No.	Filter Brand	Total Coliform Broth (35 C)	M-FC Broth (44.5 C)	Relative Error[a] (%)
1	Millipore	50	6	88
	Gelman	48	26	46
2	Millipore	46	5	89
	Gelman	48	21	56
3	Millipore	79	6	92
	Gelman	73	50	32

a. Relative error = $\frac{\text{count 35 C} - \text{count 44.5 C}}{\text{count 35 C}}$

One can improve the results with Millipore filters by incubating the stock culture at 44.5 C for an extended period of time. (Tab. 3) After 120 hours at 44.5 C the recovery on Millipore went from 13 percent to 60 percent. Please notice that the number of viable cells decreases with time but the ratio of those countable at 35 C to those at 44.5 C changes. It was thought that by serially diluting the culture, we could select for 100 percent recovery.

TABLE 3. RECOVERY DATA FOR E. COLI GROWN AT 44.5 C.

		No. of E. coli cells per 100 ml[a]		$\frac{FC}{TC} \times 100$
Tube no.[b]	Incubation Time (1 hr) (44.5 C)	Total Coliform	M-FC (44.5 C)	(%)
1	24	1,180	158	13
	72	300	30	10
	96	20	9	45
	120	30	18	60
2	48	920	85	9
	72	240	62	27
	96	110	22	20
3	24	1,780	490	28
	48	510	113	22
4	24	1,440	540	38

a. Counts are given as cells per 100 ml of a 10^7 dilution.

b. Each tube is a serial inoculation of the previous tube.

If temperature alone affects the recovery, then it should be noticable without any filter. We ran standard plate counts at various temperatures and found that the count dropped as the temperature was increased above 40 C. (Tab. 4) Please note that the recovery is about 50-60 percent of that obtained at 35 C.

We have tried to improve the technique by lowering the incubation temperature. At 35 C good differentiation was obtained between E. Coli and E. aerogenes. (Tab. 5) The same would probably be true at 40 C with Gelman membranes and we recommend that this be field tested by those who are set up to perform extensive field trials.

Let me briefly summarize our conclusions.

1. Incubation of E. coli at 44.5 C destroys the viability of at least 40 percent of the cells, even in the absence of a membrane filter.

TABLE 4. COMPARISON OF POUR PLATE RECOVERIES OF E. COLI* ON PLATE COUNT AGAR AT VARIOUS TEMPERATURES.

Temp	Average Count	No. of Plates
30 C	142	2
35 C	142	2
41.5 ± 0.5 C	94	2
44.5 C	70	2
35 C	640	4
44.5 C	470	4

* E. coli #11775

TABLE 5. DIFFERENTIATION OF E. COLI AND E. AEROGENES BY M-FC METHODS AT 35C.

		No. of cells per 100 ml	
Experiment	Culture	TGY Broth (35 C)	M-FC Broth (35 C)
1	E. coli	58	59
2	E. coli	51	48
3	E. aerogenes	59	58[a]
4.	E. coli	––	73
	E. aerogenes	––	154[a]
	Mixture (I:I)	––	230[a]
5.	E. coli	––	156
	E. aerogenes	––	253[a]
	Mixture (I:I)	––	389[a]

a. E. aerogenes colonies were cream to light green in color.

2. Use of the Millipore membrane inhibits another 50 percent of the cells. This is probably a heat soluble factor.

3. Previous incubation on Millipore membranes at 44.5 C would select for mutants capable of growing under these conditions.

I believe that a test strain of E. coli should be established. I also believe that counts of this culture on plate count medium at 44.5 C should equal the counts on the same medium at 35 C. Until they do, I do not feel that the M-FC Procedure should even be considered.

Question and Answer Session

Grasso: You made mention of your comparison studies of the fecal coliforms on Gelman and Millipore filters, and mention of numbers that were recovered. You didn't make any mention of colony size or characteristic. Did you detect any differences on the two filters at the higher temperatures?

Hufham: Yes. There is a tremendous difference. At 35 C, colonies were about the same size. When you get to 44.5 C, you find a lot of variation. You find pinpoint colonies and, if you let the culture incubate a little more, you sometimes can see additional colonies to count that weren't there previously. I don't know what causes that.

Grasso: The reason that I mention it is because, as I will state in my paper, we found marked differences at 44.5 C in the characteristics of fecal coliform colonies on the Gelman and Millipore filters, as far as size and color are concerned. I think that in addition to all the physical characteristics and media problems, there is also another problem; the actual definition of a fecal coliform.

Hufham: Let me answer that by saying we came across so many variable we didn't feel we could make a good solid study. We tried to eliminate as many variables as possible. We tried to get rid of media that seems to have a problem. We tried to get rid of the membrane and we tried various cultures to see if they grew at one temperature or another. We ended up in frustration.

Ginsburg: I want to make a comment about the rosolic acid. We found that early in our use of M-FC tests we also had a problem. But now we do not incorporate rosolic acid and get comparative results.

OPTIMUM MEMBRANE STRUCTURES FOR GROWTH OF FECAL COLIFORM ORGANISMS

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ABSTRACT

The purpose of this study was to determine the optimum membrane filter structure and characteristics for recovery of coliform organisms. Additionally, other factors such as sterilization method and membrane composition were examined. Fecal coliform growth tests with varied samples indicated that the most critical factor in recovery was surface pore morophology and not other factors previously suspected. Fecal coliform counts showed a dramatic increase with increasing surface opening sizes. Membrane structures with surface openings large enough to surround the entrapped bacteria are required for optimum growth of fecal coliform organisms. Maximum fecal coliform recoveries are obtained using membranes composed of mixed esters of cellulose exhibiting a surface opening diameter of 2.4 μm and a retention pore size of 0.7 μm.

INTRODUCTION

Since its introduction as a tentative method for coliform enumeration in the 10th Edition of Standard Methods in 1955, the membrane filter has gained wide acceptance not only for total coliform, but also for fecal coliform, total bacteria, and a wide variety of other bacterial tests. The unique advantage of the membrane over other test methods is its ability to concentrate and localize bacteria from large samples. Hence, the membrane increases the sensitivity of quantitative bacteriology into the range well below one organism per ml. Once the bacteria are localized, the membrane provides a structure for counterdiffusion of nutrients and metabolic products as well as "hospitable" growth environment. In these functions, the membrane differs little from the earlier pour and streak plate methods.

The earliest technique for bacteriological analysis with membrane filters involved direct microscopic examination of bacteria trapped on the membrane surface. Here, the optimum structure required pores smaller than the organisms being trapped for examination so that they would lie in a single microscopic plane. This **surface planar retention** facilitated finding the organisms under high power microscopy. The above requirements evolved naturally to the practice of retaining organisms on the membrane surface for various culture techniques. At that time, not much thought was given to developing an optimal membrane structure for colony growth.

The ideal characteristics of a membrane for quantitative bacteriology would appear to be pores small enough to retain bacteria but open enough to provide paths for liquid transports, and a "hospitable" surface growth. However, upon examining the variety of bacterial methods utilizing membranes, one finds a considerable range of bacteria sizes, types, and metabolic requirements. These considerations led us to wonder if it was possible to develop membranes which would be especially favorable for the growth of particular types of organisms, such as the coliform group.

The critical step in development of a colony from a single bacterium is the onset of cellular division, and it is not unreasonable to expect that this delicate process could be affected by the extent and nature of the contact of the organism with the solid, and the extent and thickness of the nutrient film surrounding the organism. Further, nutrient supply by diffusion of medium and removal of subsequent metabolic waste products must be a function of membrane structure and pore morphology.

With these factors in mind, we began this study with the objective of defining the optimum membrane structure for growth of coliform bacteria.

MATERIALS AND METHODS

Membranes were obtained from a variety of commercial sources and from our experimental membrane development activities. The surface structures of these were characterized using a Coates & Welter CWICSCAN 100-4 scanning electron microscope. Before observation, the membranes were coated with a 100 - 200 A layer of gold.

Fecal coliform and total coliform determinations were performed in accordance with Standard Methods (1), Sections 408 A and B with the following modifications: To achieve the closest possible similarity between membrane tests and streak plate control, the membranes were plated on a 0.34 cm thickness of agar medium in 47 mm petri dishes; each streak plate was prepared by spreading a 0.1 ml aliquot of sample onto a 0.34 cm thickness of agar in a 90 mm dish. The reason for using a controlled thickness of agar is that we had found, in earlier experiments, that fecal coliform recovery is a function of agar thickness (2).

M-FC Agar and M-Endo Agar were obtained from the BioQuest Division of Becton, Dickinson, and Company. Plates were stored at 5 C and were used within 48 hours of preparation. Fecal coliform plates were incubated at 44.5 C ± 0.2 C in Blue M waterbaths equipped with calibrated recording thermistors. Total coliform plates were incubated at 35 ± 0.5 C in circulating air incubators.

Most of the water samples were untreated sewage, obtained from the masher section of the Billerica, Massachusetts, Sewage Treatment Plant. River samples were also used. Samples were stored at 5 C and were used within 30 hours of collection.

Some refinements of technique were needed to allow us to run experiments involving large numbers of samples. Initially, it was found that noticeable die-off occurred in 15 minutes when the source water was diluted with phosphate buffer. The use of 0.1% buffered peptone, however, stabilized the count for a period of one hour (2). We also found that it was important to restrict the time between plating and incubation to 15 minutes or less. The complete procedure was then as follows: A preliminary count was obtained when the sample was taken. The following day, a dilution was prepared to give a count of 200 – 1,000 bacteria/ml, using buffered peptone diluent. The diluted sample was mixed for 30 minutes on a mechanical shaker. Then groups of about 18 membranes and 9 streak plates were prepared from 0.1 ml aliquots, plated, and incubated. This was repeated throughout the experiment. Using this method, up to 100 membranes plus associated streak plate controls could be run within the one hour limit. To confirm fecal coliforms, typical blue colonies were transferred into Lauryl Tryptose broth and then into EC broth.

Surface Pore Morphology

Membrane filter structure can be characterized by several parameters. The **retention pore size** is a measure of the smallest particle which is retained by the structure, and is best measured by direct determination of passage of particles (or microbes) of known size. This technique is described by Rogers and Rossmoore (3).

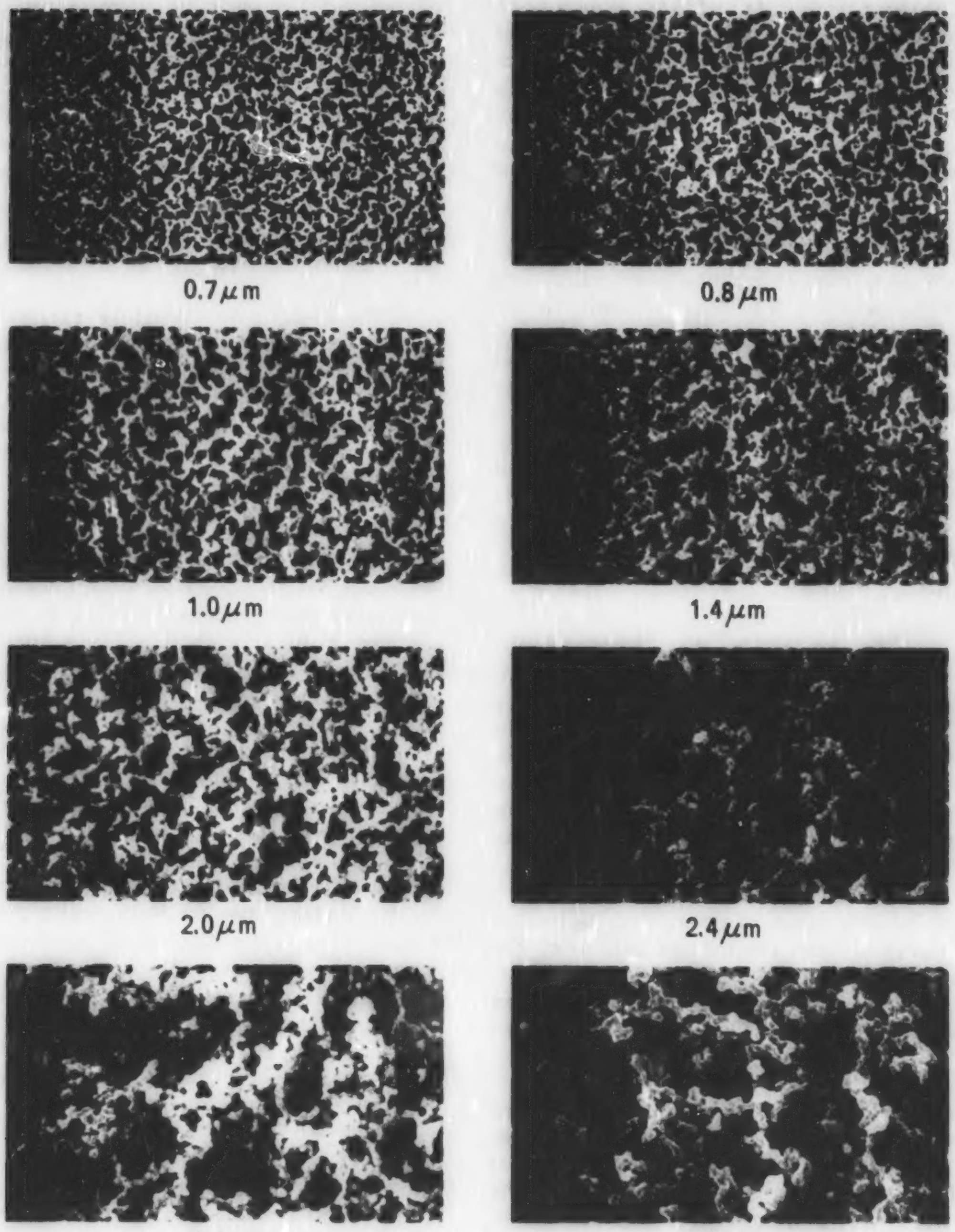
In the present investigation, we were interested not only in bacterial retention but also in how the bacteria are situated on the membrane. It is reasonable to expect that the environment of retained bacteria depends on the retention pore size as well as the structure of the surface layer in which they are retained.

Figure 1 gives scanning electronphotomicrographs of a series of eight membranes made from mixed esters of cellulose. The photomicrographs show similar structures which differ only in the size of the openings. In each photomicrograph relatively large surface openings were characterized by the **surface opening diameters** reported on the Figure. These were determined by direct measurements on each photomicrograph, or in the case of the smaller size openings by measuring enlargements of the photomicrographs. The **retention** characteristics of these membranes for coliform organisms were determined by passage tests, as described in the following section.

In summary, the way in which bacteria are situated on a membrane is determined by a new parameter, the **surface opening diameter**, which is observable from scanning electron photomicrographs. The retention of bacteria is determined by the more familiar **retention pore size**, which is found from passage tests.

Results

Figure 2 shows fecal coliform counts on the series of membranes described above. There is a



0.7 μm

0.8 μm

1.0 μm

1.4 μm

2.0 μm

2.4 μm

3.0 μm

4.0 μm

Figure 1. Scanning Electron Micrographs of a Series of Mixed Ester of Cellulose Membranes. Numbers shown are Surface Opening Diameters.

5 μm

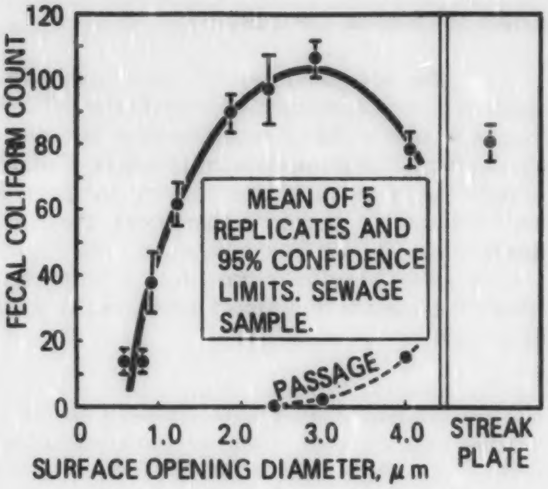


Figure 2. Fecal Coliform Count versus Surface Opening Diameter.

remarkable increase in counts at surface opening diameters between 1.0 and 2.0 μm. The decrease in counts at the largest opening size is evidently due to passage of organisms through this very coarse structure. The dotted line labeled "passage" was obtained by re-filtering the effluent through a bacterial retentive membrane and plating this membrane on M-FC Agar in the usual way. On the basis of both growth and passage tests, the optimum membrane structure was determined to have a 2.4 μm surface opening diameter with smaller (fecal-coliform retentive) voids of approximately 0.7 μm internally. Results of this plus three other fecal coliform runs are given in Figure 3. In all four runs, the abrupt increase in recovery at a surface opening diameter of 1.0 to 2.0 μm is evident, with the optimum structure - i.e., zero passage and optimum growth occuring with 2.4 μm surface openings.

Typical blue colonies were picked for confirmation from the 0.7, the 1.4, and the 2.4 μm surface opening membranes. The ratios of confirmed/picked were 18/20 for the 0.7, 19/20 for the 1.4, and 17/20 for the 2.4 μm surface opening membrane.

Figure 4 presents the results of two total coliform experiments on the same series of filters. Here, a light effect may be observed occurring only at the smallest and largest surface opening sizes.

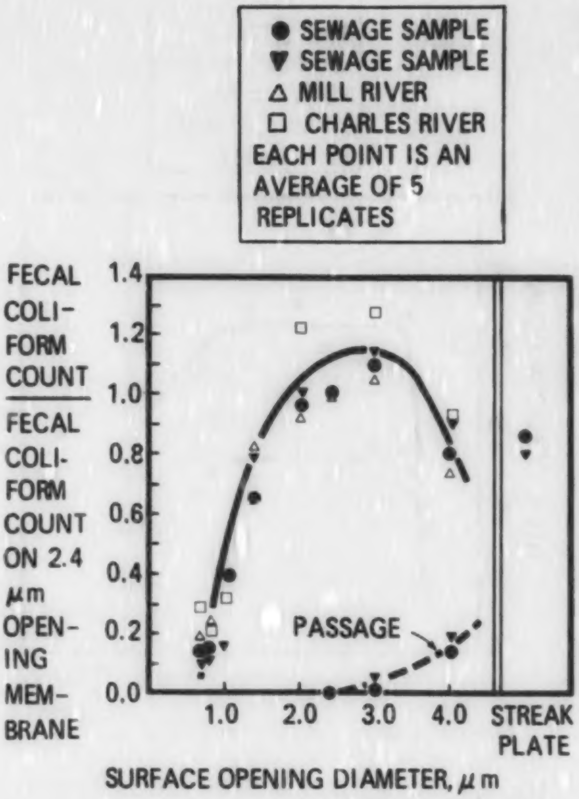


Figure 3. Normalized Fecal Coliform Counts versus Surface Opening Diameter.

To compare the total and fecal coliform effects more directly, a culture was prepared from a typical fecal coliform colony from the Mill River source. This was run on M-FC agar and M-Endo agar using the same series of samples, all from the same dilution. Results are presented in Figure 5. Here, the total coliform test shows only a very slight surface opening size effect while the effect is considerably more evident in the fecal coliform test. Evidently, while the fecal coliform test requires a surface opening diameter of 2.4 μm for optimum growth, the total coliform test is less demanding and is performed well on membranes of surface opening diameter in the range, 1 to 3 μm.

At this point, it appeared that surface opening diameter was definitely a primary determinant of fecal coliform recovery. However, other factors, such as chemical composition and methods of sterilization, remained to be investigated.

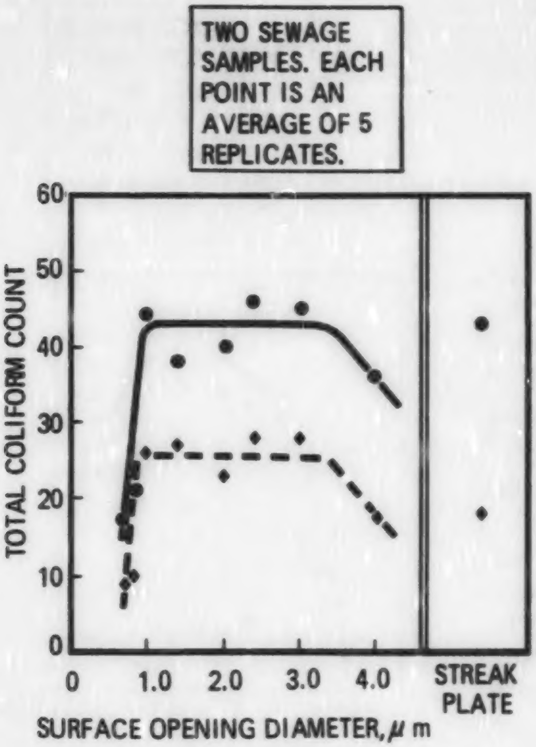


Figure 4. Total Coliform Count versus Surface Opening Diameter.

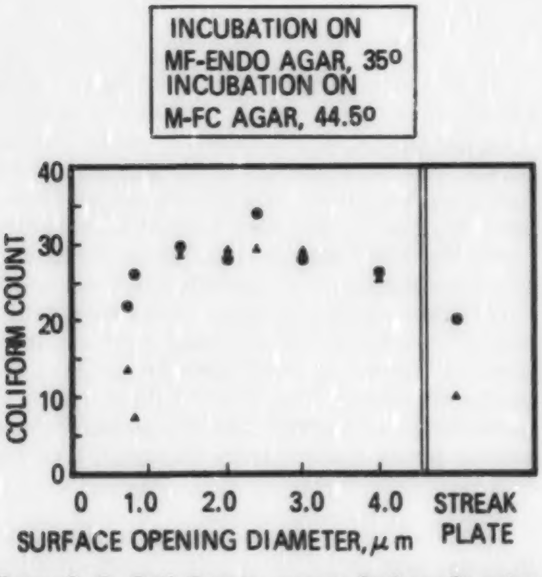


Figure 5. E. Coli Counts versus Surface Opening Diameter. A Comparison of Total and Fecal Coliform Tests.

Effect of Chemical Composition

In the foregoing set of tests, membranes employed were composed of mixed esters of cellulose. A second series of experiments were designed employing cellulose acetate membranes. Cellulose acetate has a much smaller affinity for proteins, and presumably bacteria, than does the mixed esters material used in the previous tests. Thus, if surface adhesion affects growth, a difference between the acetate and mixed esters results should be evident.

In the next experiment, recovery on the 2.4 μm (surface opening) mixed cellulose esters membrane was compared with that of a 3.8 μm (surface opening) cellulose acetate membrane. In addition, an experimental non-cellulosic membrane composition having a 3.0 μm diameter surface openings was included. Results are given in Figure 6. Here, we have plotted the actual counts on each of five replicates, and the passage count obtained by re-filtering the effluents from each. The results show very little difference in count between the three membrane compositions. These results suggest that

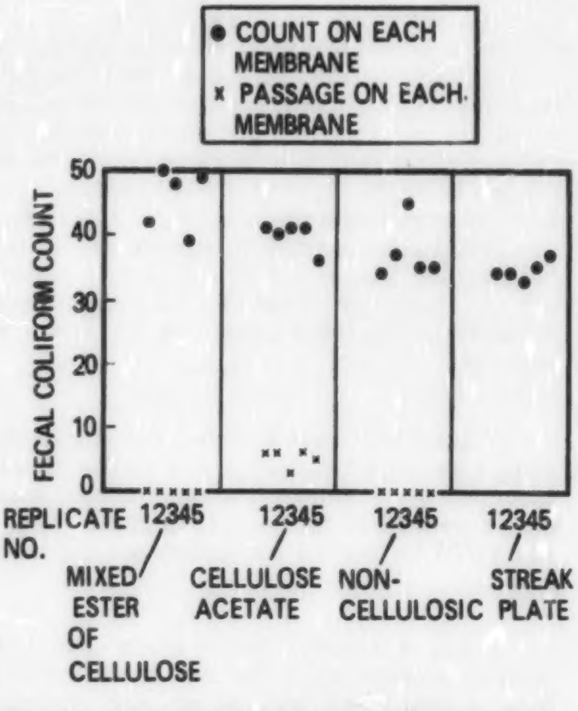


Figure 6. Fecal Coliform Count on Membranes of Three Different Compositions.

membrane composition is not an important factor in fecal coliform recovery.

Effect of Sterilization Method

Several authors (4, 5) have suggested that bacterial recoveries may be affected by the method of sterilization. They did not, however, present data derived from comparing identical membranes, where the only variable was the method of sterilization. To test for possible sterilization effects, membranes were selected from the group exhibiting optimum growth characteristics (2.4 μm surface openings). These membranes were then divided into four groups using random sampling techniques. One group was left unsterilized, one was autoclaved at 121 C for 15 minutes, one was exposed to ethylene oxide using a standard sterilization cycle * and was aerated three days, and the fourth group was sterilized by irradiation at a dose of 1.0 megarads using gamma rays from a cobalt 60 source. Mean counts and 95% confidence limits on the means are given in Table 1, and are discussed in more detail in our other paper (2). There are no significant differences between counts on the unsterilized membranes and counts on the membranes sterilized by the three methods used.

TABLE 1 EFFECT OF STERILIZATION ON MIXED CELLULOSE ESTER MEMBRANES HAVING 2.4 μm SURFACE OPENING DIAMETER

	Total Coliform Count**	Fecal Coliform Count**
Unsterilized	42 ± 6	99 ± 9
Ethylene Oxide Sterilized	44 ± 6	103 ± 9
Autoclaved	38 ± 6	108 ± 9
Irradiated	40 ± 6	94 ± 8
Streak Plate	50 ± 6	82 ± 6

** Two different sewage samples were used. Each mean is an average of five replicates.

Discussion

The data collected to this point strongly suggest that neither chemical composition nor method of sterilization has any significant effect, but that the primary determinant of fecal coliform growth on a membrane filter is that of the surface pore morphology (specifically with respect to the size of upper surface openings).

We speculated that since surface effects are strongest at surface void sizes which are close to coliform dimensions, some sort of fit of the organism into the pore might be required for optimum growth. In particular, the mechanism of the effect could be that organisms which are deposited on very fine surface structures are incompletely surrounded by nutrient, while ones that fit into surface openings can be cradled below the level of nutrient that is drawn up by capillary forces. Because of evaporation, an incompletely surrounded bacterium might be subjected to a locally hypertonic solution, with resulting plasmolysis and death. This effect would be particularly evident at the elevated temperature (44.5 C) of the fecal coliform test.

To test this hypothesis, three methods of supplying nutrient were compared. The 0.7 μm and the optimum 2.4 μm surface opening cellulose ester membranes were used. One set was plated in the standard manner, one set was plated face down on the M-FC Agar, and the third set was plated right side up with 2.0 ml of M-FC Agar overlayed onto each membrane. Results are summarized in Table 2.

Due to the confluence of colonies, accurate counts could not be obtained from the membranes placed face down. However, it was clear that the number of colonies on membranes having the smaller surface openings (0.7 μm) was substantially increased by placing face down. Overlaying these membranes gave a dramatic increase in counts. The increase in growth thus seen from inverting the filter, plus the close agreement in counts of the two membrane groups when the lower yield filters were overlayed with nutrient, gives strong evidence that complete nutrient coverage of the organisms is required and that this is achieved only with larger surface opening sizes.

During the comparison testing of the membranes for the 0.7 μm and 2.4 μm surface opening groups, some additional benefits were noted rela-

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* The cycle used a two hour exposure of 12% ethylene oxide at 130 F and 60% relative humidity (6).

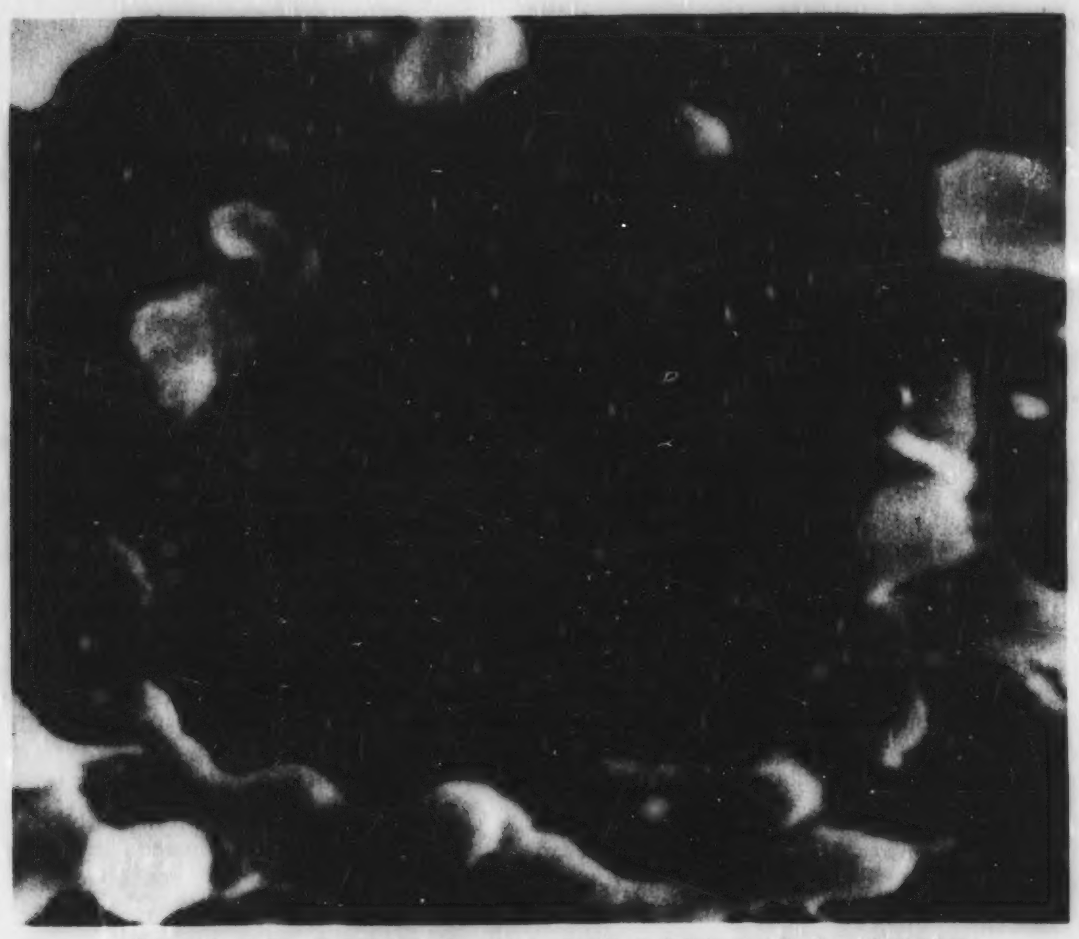

Figure 7. A Fecal Coliform Organism Cradled within the Surface Opening Diameter of a Type HC Filter (15,500X).

TABLE 2 EFFECT OF PLATING METHOD AND PORE SIZE FECAL COLIFORM TEST, SEWAGE SAMPLE

Method of Plating	Mean Counts and 95% Confidence Limits Membranes with 0.7 μm Surface Openings	Membranes with 2.4 μm Surface Openings
Membrane Plated in Standard Manner	14 ± 3	44 ± 10
Membrane Inverted on Agar	Approx. 30	Approx. 45
Membrane Plated and Overlayed	46 ± 7	53 ± 8
Streak Plate	35 ± 5	

tive to the latter. These predictable, but nonetheless important, phenomena were an increase in the flow rate through the membrane, an increased diffusion rate of media to the membrane surface and, significantly, increased capacity to filter large volumes of water particularly those where algae or other collodial turbidity would otherwise limit the sample size.

In summary, the factors expected to have an effect on fecal coliform recovery were investigated. The only one showing a significant effect was that of surface pore morphology. The evidence suggests that unlike other organisms, fecal coliforms specifically must be cradled slightly below the membrane surface for optimum recovery. This suggests an optimum membrane structure with surface pores slightly larger than the fecal coliform organisms, but with internal bacterial retentive pores. See Figure 7.

Until now, membranes recommended for bacterial testing have been specified by a **retention pore size** of 0.45 μm. Typical 0.45 μm retention membranes have **surface opening diameters** of 1 to 2 μm. As can be seen in Figures 2 and 3, a slight shift of position on the curve in the range of 1 to 2 μm surface openings can have a large and significant effect on recovery.

Since membranes of different manufacturers, all having 0.45 μm retention size, may exhibit differences in surface morphology (i.e., in relative surface opening diameters) they may also exhibit considerable differences in fecal coliform recovery.

A change to the optimum 2.4 μm surface opening size will not only provide higher fecal coliform counts, but will also lead to a smaller sensitivity to small differences in surface morphology.

For the **total coliform** test (Figure 4 and 5), however, membrane performance is not sensitive to surface morphology (except in the range below 1 μm surface opening size). The new 2.4 μm surface opening/0.7 μm retention pore size membrane developed in this work should be regarded as an improvement for **fecal coliform** tests, and may also be used for total coliform with results equivalent to 0.45 μm retention membranes.

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5. Dutka, B.J., M.J. Jackson, and J.B. Bell. Comparison of Autoclave and Ethylene Oxide-Sterilized Membrane Filters Used in Water Quality Studies. Appl. Microbiol., 28, 474, 1974.
6. Kereluk, K., and R.S. Lloyd. Ethylene Oxide Sterilization. J. Hospital Research, 7, 7, 1969; Cycle is shown in Figure 32, p.67.

QUESTIONS AND ANSWERS

Hufham:	We did a study which we are not quite through with yet, in which we washed membranes in weak sodium bicarbonate at 50 C, then in distilled water, and then reautoclaved. We tried to see if we could get an inhibitor out, and got an increase of 50% on our counts with your particular filter. We were a little afraid however, that we were distorting the filter. I wonder if you would comment on a procedure like this; might we be making the pore sizes bigger as you describe in your paper?
Sladek:	It's possible that something like boiling or strong autoclaving can have a slight effect on the surface morphology of the membrane.
Ginsberg:	I, and perhaps others, don't quite understand what the difference is between the pore size and the surface opening size?
Sladek:	I am glad that you asked that, because I would like to make it absolutely clear. With filtering to remove particles or bacteria we are interested in retention of particles and bacteria. That is the way we characterize our material, by retention pore size.

The way you measure retention pore size is by retaining something. That is the only certain way to characterize a membrane for retention, by performing a passage test. Generally these are done with microorganisms since the size distribution is quite narrow. I can give you some references to the procedure. This is the retention pore size; it's something that is experimentally determined. You can only speculate how this relates to the structure. You can't really make a microscopic study using a scanning electron microscope and say what the retention pore size is. I would like to view the retention pore size and the surface morphology characterized by surface opening diameter as two separate and independent parameters characterizing a membrane. In fact, they aren't entirely independent. As you see, one is constrained as to what you can manufacture, so that as you increase the surface opening diameter you also increase the retention pore size, generally speaking, for any given type of membrane.

Bordner: Your remarks are certainly interesting and I think you have given us a lot of new information. I don't know whether to go home and pour selective agar over all my membranes or throw them all out and buy this new proposed material that you describe. Do I understand that these are experimental materials with the larger surface porosity that you are talking about, not the ones that I have been buying recently?

Sladek: These materials that I showed you were made specifically for this test. Now they do have a lot of similarities to present 0.45 μm membranes.

Bordner: Then are we talking about the possibility of a membrane filter formulated specifically for fecal coliforms?

Sladek: Yes, indeed we are.

Grasso: If you give the right answer I might not come back tomorrow and give my paper. Do you feel that with this increased pore size the temperature effect and all the other variables mentioned today are overcome? In other words, you could use this new filter with the larger surface pore size and with the M-FC broth and overcome these difficulties and problems?

Sladek: Yes, I think you have asked me if this is the cause of all the controversy?

Grasso: Right.

Sladek: I think to a large extent, yes. In the figure that I gave on the fecal coliform count versus the surface opening diameter, this slope was very steep on the left. This means that different membranes that are manufactured by various companies striving for the same retention pore size were not controlling the surface morphology directly. The surface morphology was down in a range where it was a very sensitive parameter with regard to the fecal coliform test. I think we can look forward to a period when all those differences will go away, because now we move up further on the curve towards the peak.

Grasso: Are these experimental membranes going to be available?

Sladek: As far as I know they will be very shortly.

Seidenberg: How much vacuum did they use to pull those samples through, and what is your recommended vacuum?

Sladek: Let me refer this question to our bacteriologist, Mr. Dawson.

Dawson: I don't think vacuum has any tremendous effect on whether or not an organism is impinged deeper into a smaller pore or remains near the surface. It's been known for a long time that the gram negative microorganisms are extremely sensitive to hypertonic solutions. Indeed this is the recommended method for preparing protoplasts from gram negative organisms. I believe in our laboratory we normally use something like 14" vacuum for running samples. I've been working with membranes since about 1955 and I haven't seen any effects that were due to vacuum.

Brezenski: I think I recall one paper that discusses cavitation and the effect of vacuum on coliforms, so I don't quite believe what you said.

Levin: Two questions really – one is on the steep part of the curve, where you were talking about the surface effects. I didn't really catch it. When you boil or autoclave you go up in terms of improving recovery or do you go back down in terms or decreasing recovery after treating them in your own laboratory?

Sladek: We have found very little effect of either procedure. I showed you some data on autoclaving.

Levin: It really doesn't make any difference?

Sladek: No.

Levin: The second question is only indirectly related but since you're a statistician I thought that I would put you on a spot, if I could. You've given data in two papers and your counts have ranged from as low as 9 or 10 up to about 120 for the average count and yet we think of 20 to 80 as being the optimum numbers. I'm wondering where you would draw the line? For instance, if I'm doing a one hundredth, a tenth, one ml and 10 ml, and my 10 ml averages 12, and my 1 ml averages 100, which one do I believe and have the most faith in?

Sladek: Yes, let me comment on that. I'm sure that you realize in terms of the statistics that you want to avoid low numbers, so it is to your advantage to get up into the higher numbered range. We have not established for certain whether there is an upper cutoff beyond which you should go. We have had good results going up to about 140. I mean "good" in the sense that the scatter didn't increase extraordinarily. I've seen data up beyond 200 where the scatter was really terrible and I am not sure how safe it is to conclude that above 200 something goes wrong . . . perhaps someone else has some information on that.

Litsky: Did you examine brand X for cavitation and if so, what did you find?

Sladek: You are asking if we examined other brands of membrane filters so far as their surfaces go? Let me answer that in this way. I would like to refer to my earlier paper. I stressed there the idea of random sampling. Random sampling is a very important concept in not producing bias in your data. We have tested what I will call a few boxes of competitor's membrane filters. I will not tell you the results because I know that this does not represent a random sample of their production. I can tell you the results that we have obtained on Millipore filters because we are very careful to take a representative random sample of our production and we base results that we report on that. I can't really comment in public about other manufacturers' filters because I don't have a proper random sample of their production.

Litsky: May I take my prerogative as the chairman and ask any other representatives, Gelman, Johns-Manville or anyone else, if they examined their filters and observed the cavitation effect?

Sladek: Please don't call it a cavitation effect.

Litsky: Litsky stands corrected.

Brezenski: I was going to mention that cavitation isn't what we are talking about here. I want to get back to surface morphology and the reasons that you gave why there was no effect on the recovery of the total coliforms, yet there was a decrease in recovery. You assumed this because of the surface morphology and that fecal coliforms stay on the surface and don't get deep down into the layers where they can get more medium. I haven't seen any data that you've presented which shows specifically that surface morphology effects the fecal coliform and not the total coliform. Do you have any data which shows the total coliforms in the upper layers and the fecal coli, or E. coli in the bottom layers? This seems to be the crux of your explanation.

Sladek: No. This is not the crux of my explanation.

Brezenski: Oh, I am sorry. Would you clarify that?

Sladek: The organisms are of course the same size. They are from the same group. We are speaking of what happens to an organism that is filtered on a very fine surface structure, and ends up "on a moutain top". The explanation was that because of evaporation, having to do with dehydration rates, vapor pressures, etc. a fecal coliform organisms may be in contact with a pool of nutrients, which will shrink in size and consequently become concentrated, or locally hypertonic, causing plasmolysis of the organism. If this is indeed the mechanism, you would expect it to be much less pronounced at a lower temperature where the evaporation rate is lower. In trying to tie all of these things together, we have shown rather conclusively that it is the way in which nutrient is supplied that is the origin of the effect. We did this by turning the membrane over and pouring agar on top of it, etc. We have also shown that the effect is very strong in the fecal coliform tests, but rather weak in the total coliform test. The way we bridged the gap and put all this back together has to do with the evaporation rate.

Winter: I just have one quick question. I can't really quite understand evaporation as the reason, or should I say the hypothesis, because we are dealing with a saturated humidity chamber whether you are on M-FC in a bag or in a super tight fitting plate. I wonder whether this really has an effect. Aren't we really reaching a bit to explain why something happens that we don't know anything about?

Secondly, although you may not have tested many Gelman or other filters, it would seem ironic that some of the other manufacturers are ahead of you, because they, by some process, were able to prepare a membrane with larger surface openings, hence large recoveries. If we just look at the summary of results they seem to conclude that under present manufacturing techniques, Gelman seems at least to have given higher recoveries by the majority of experimenters and no one has explained this fact. We may have some random error occuring, and we know we have systematic error in everyone's work, but this is randomized, by the number of manufacturers putting out membranes and the number of people doing the work, when we have had perhaps as many as 50 people coming up with results which all tend to point in the same direction. I am wondering aren't we reaching a bit at this time?

Sladek: Let me respond to your first question. Aren't we reaching a little in the explanations? Yes, indeed we are reaching in the explanation. Concerning your comment that it is a closed system, with respect to water vapor, there are always some small gradients in these systems and water vapor does not move around inside the plate, so that is about the only support that I can give to the idea that small changes in concentration can occur.

Again, with regards to your other question, I would have to defer any comments on what the real status of different membrane manufacturers' membranes are, pending a study. I believe the D-19 round robin study has been completed and I certainly would not accept your statement that everyone agrees that this kind of filter is better than that kind, because everyone doesn't.

A COMPARISON OF MEMBRANE FILTERS AND MEDIA USED TO RECOVER COLIFORMS FROM WATER

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ABSTRACT

Many laboratories involved in water analysis are using membrane filtration methods for the enumeration of pollution indicator organisms in water. The Ontario Ministry of Health, Laboratory Services Branch, analyzes approximately 350,000 water specimens annually, almost exclusively by membrane filtration. We have observed that there are pronounced differences in the abilities of the filters produced by various companies to recover coliforms. This paper reports the results of an investigation which evaluated three brands of membrane filters. Seven of our laboratories participated in this study. Parallel analyses for total coliforms from routine water samples were performed using filters supplied by the Johns-Manville Company of Canada (045 MO 47SG), the Millipore Corporation (HAWG 47SO) and the Sartorius Company (11456). Statistical evaluation of the results indicated that the Johns-Manville and Millipore filters were equivalent and much superior to the Sartorius filters for the enumeration of coliforms from water.

LES Endo agar is the only solid medium recognized by Standard Methods for the Examination of Water and Wastewater (13th ed. 1971) for the direct recovery of coliforms from water. M-Endo broth media are also recognized by Standard Methods for use in membrane filtration; however, it is recommended that these broth media preparations be used with sterile pads. This latter procedure as outlined in Standard Methods, adds an additional time factor to the processing of each water specimen which would present difficulties to a high volume laboratory such as ours.

The relative cost of these two types of Endo preparations as well as the problems created by the recently experienced shortages of Endo based products prompted us to compare LES Endo agar with various M-Endo broths with agar added for coliform analyses.

INTRODUCTION

Specifications for membrane filters used for the bacteriological analysis of water are presented in the 13th edition of "Standard Methods for the Examination of Water and Wastewater".

All manufacturers of membrane filters claim or intimate in product advertising that their filters meet the criteria specified in Standard Methods for bacterial recovery. Recent investigations indicate that, despite manufacturers' claims, there exists considerable variation among commercial brands of membrane filters in their ability to recover coliform and faecal coliform organisms from water. There is, however, some disagreement concerning experimental designs and statistical evaluations used in these studies. Consequently, any general conclusions to be drawn from such investigations must be guarded.

The Laboratory Services Branch of the Ontario Ministry of Health analyzes more than 350,000 water specimens annually, almost exclusively by membrane filtration. Our laboratory personnel had also observed inconsistencies in coliform and faecal coliform enumeration on membrane filters produced by various companies. As a result of these observations we carried out a series of investigations to quantitatively compare coliform and faecal coliform recoveries on three brands of membrane filters — Johns-Manville, Millipore and Sartorius.

MATERIALS AND METHODS

The comparative evaluations were done in three phases. Phase 1 was a preliminary field study involving five regional laboratories. Phases 2 and 3 were more rigidly controlled investigations conducted solely in our central laboratory.

Membrane Filters:

The Millipore filters (Catalogue No. HAWG-047SO) and the Sartorius filters (Catalogue No. 11456) used throughout the investigation were obtained from the Ministry of Health Laboratory's stock supplies. The Johns-Manville filters (Catalogue Nos. 045M047SG and 045M047LG) were supplied by the company.

Three different lot numbers of the three filter brands, pre-sterilized by ethylene oxide, were included for comparison in the first and second phases of this investigation. For the third phase, unsterile Johns-Manville filters were obtained and autoclaved in our laboratory.

Source of Cultures:

In the preliminary phase 1 of the study we used routine water samples. Appropriate dilutions of the samples were filtered in duplicate for total coliforms only. All the participating laboratories did not stock the same brands of filters. Three laboratories compared Johns-Manville with Sartorius filters and two others compared Johns-Manville with Millipore filters.

Similar routine water samples were used for the second phase of this study. After being analyzed by our routine procedure for total and faecal coliforms, these water samples were refrigerated overnight. The following day, those samples having at least 20 faecal coliforms per 100 ml were selected for further processing. Ten ml of each of these samples were added to 10 ml of double-strength MacConkey broth in screwcapped fermentation tubes. Following incubation for 24 to 48 hours at 35C, two loopfuls of each positive broth culture were subcultured into EC broth. The EC broths were incubated at 44.5C for 22 to 24 hours. Five replicate filtrations of a dilution of each EC broth culture, standardized by optical density, were performed for both total and faecal coliforms for each of the three brands of filters.

In phase 3 of the study, we collected eight, one-litre samples of water from a known polluted surface source, the Humber River, over a one week period. On the day of collection, a prescreening membrane filtration was done on each sample, to determine coliform and faecal coliform densities. Based on the screening densities, appropriate test dilutions of the refrigerated samples were prepared to provide 20 to 80 total coliform colonies and 20 to 60 faecal coliform colonies per filter. Ten replicate filtrations per sample per brand of filter were completed on each water sample for total and faecal coliforms.

Cultural Techniques:

Throughout the study, M-Endo MF broth (Difco) with 1.5% agar added was used for total coliform recovery, and M-FC broth base (Difco) with 1.5% agar added was used to culture faecal coliforms. These solid media were prepared in 15 x 150 mm plastic petri plates, which accommodate 5 filters. Incubation times and temperatures were as specified in Standard Methods. The M-FC plates were heat sealed in waterproof plastic bags before being immersed in a constant temperature water bath at 44.5C.

Statistical Analysis:

The Students t test for comparison of means was used to evaluate the results of the preliminary field study. Eighty-two comparisons between the Johns-Manville and Sartorius filters were tabulated. Sixty eight comparisons between Johns-Manville and Millipore filters were analysed.

Analysis of variance (ANOVA) was applied to the results of phases 2 and 3 of this investigation. When the F ratio indicated a significant difference in the means at the 5% significance level, a multi-mean comparison test (the Tukey Test) was used to determine where the difference occurred.

RESULTS AND DISCUSSION

Our results clearly demonstrate that variations in experimental design can lead to very different conclusions regarding the superiority of one MF brand over others. Tables 1 and 2, summarizing the statistical analysis of the preliminary field study, show that the Johns-Manville filters were superior to Sartorius filters but equivalent to Millipore filters for total coliform recovery. But the results of phase 2 conflicted with this conclusion. Recovery of faecal coliform isolates on m-Endo medium (Table 3) concluded that the three brands

Table 1: **Total coliform recovery from routine water samples in three laboratories with Johns-Manville and Sartorius membrane filters.**

	Johns-Manville	Sartorius
No. Comparisons	82	82
Means (x̄)	14.9	8.1
Standard Deviation	15.50	11.94
t-Test Analysis	t = 3.17 (t .05, 162 = 1.92)	

of filters were equivalent; but, recovery on M-FC medium concluded that Johns-Manville filters were superior to both the Millipore and the Sartorius filters (Table 4). There was no significant difference in faecal coliform recovery between Millipore and Sartorius filters.

We attempted to resolve this conflict of data by the experimental design of phase 3 using natural water samples. Statistical analysis of total coliform recovery by the three filters (table 5) supports the findings of the preliminary study, i.e. the Johns-Manville filters were superior to Sartorius filters but equivalent to Millipore filters for total coliform recovery. As in the preliminary study, these results conflict with the results of phase 2. Similarly, statistical analysis of faecal coliform recovery rates from natural water samples (Table 6) indicate that there was no difference among the three brands of filters at a significance level of .05. This finding also disagrees with phase 2 of the study, which used laboratory cultures of faecal coliform isolates. Similar disagreements have been described by other investigators. Presswood and Brown (4), and Harris (3) concluded that Gelman membrane filters were superior to Millipore membrane filters for recovery of E. coli. Schaeffer et al (5) disagreed with their statistical conclusions. Schaeffer's group found when using natural water samples, that Gelman and Millipore filters were equivalent for faecal coliform recovery, but, that Gelman filters were superior to Millipore filters for total coliform recovery.

Table 2: Total coliform recovery from routine water samples in two laboratories with Johns-Manville and Millipore membrane filters.

	Johns-Manville	Millipore
No. Comparisons	68	68
Mean (x̄)	192.3	184.5
Standard Deviation	499.60	335.80
t-Test Analysis	t = .05 (t .05, 134 = 1.98)	

Table 3: **Recovery of faecal coliform isolates on M-Endo medium with Johns-Manville, Sartorius and Millipore membrane filters.**

	Johns-Manville	Sartorius	Millipore
No. Comparisons	100	100	100
Mean (x̄)	52.0	44.5	43.7
Standard Deviation	30.70	30.87	32.28

Analysis of Variance (ANOVA)

Source	SS	df	MS	F ratio[a]
Within	290885.70	297	979.31	2.14
Between	4194.06	2	2097.03	

[a] $F_{.05\infty} = 3.00$

In a recent paper, Dutka et al (2) reported conflicting results in two studies employing the same experimental design. Field samples and broth cultures of E. coli ATCC 25922 were used for comparative recoveries with autoclaved and ethylene oxide sterilized filters. The results of their first study (March 1973) concurred with findings of Schaeffer et al (5). At a significance level of .01, Gelman and Millipore filters were equivalent and superior to Sartorius filters for faecal coliform recovery; however, Dutka et al (2), also reported that both Millipore and Sartorius filters were

Table 4. Recovery of faecal coliform isolates on M-FC medium with Johns-Manville, Sartorius, and Millipore membrane filters.

	Johns-Manville	Sartorius	Millipore
No. Comparisons	100	100	100
Mean ($\bar{x}$)	40.5	30.3	33.1
Standard Deviation	32.47	26.75	28.19

Analysis of Variance (ANOVA)

Source	SS	df	MS	F ratio[a]
Within	25387.02	297	854.78	
				3.22
Between	5496.17	2	2748.08	

Multimean Comparison Test (Tukey Test)

Tukey Calculation[b]	Johns-Manville vs Millipore	Johns-Manville vs Sartorius	Millipore vs Sartorius
$(\bar{x}_1-\bar{x}_2) \pm T$[c]	+17.03 to − 2.33	+19.83 to + 0.47	+13.48 to − 6.88
Conclusion (φ = .05)	Not significant	Significant	Not significant

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[a] F $.05\infty$ = 3.00

[b] Reject H_0: $\mu_1 = \mu_2$ if $(\bar{x}_1 - \bar{x}_2) \pm T$ does not include zero

[c] T = Tukey Statistic

Table 5. Total coliform recovery from Humber River water samples with Johns-Manville, Sartorius, and Millipore membrane filters.

	Johns-Manville	Sartorius	Millipore
No. Comparisor	80	80	80
Mean ($\bar{x}$)	49.1	34.7	42.7
Standard Deviation	25.81	22.01	27.12

Analysis of Variance (ANOVA)

Source	SS	df	MS	F ratio[a]
Within	149039.18	237	628.86	
				6.62
Between	8327.48	2	4163.74	

Multimean Comparison Test (Tukey Test)

Tukey Calculation[b]	Johns-Manville vs Millipore	Johns-Manville vs Sartorius	Millipore vs Sartorius
$(\bar{x}_1-\bar{x}_2) \pm T$[c]	+15.6 to −2.8	+23.6 to +5.2	+17.2 to −1.2
Conclusion (φ = .05)	Not significant	Significant	Not significant

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[a] F $.05\infty$ = 3.00

[b] Reject H_0: $\mu_1 = \mu_2$ if $(\bar{x}_1-\bar{x}_2) \pm T$ does not include zero

[c] T = Tukey Statistic

inferior to Gelman filters for total coliform recovery. The results of their second study (June 1973) suggested no significant difference among the filters for faecal coliform enumeration and only slight differences for total coliforms.

In addition to the quantitative aspects of our comparative membrane filter study, we noted certain undesirable qualitative features of some filters. Coliforms on ethylene oxide sterilized Johns-Manville filters, when grown on M-Endo-MF agar, did not always form discrete colonies. Instead, the colonies spread to the grid lines, giving them a square appearance, which we referred to as "plaquing." Even with this plaquing, the Johns-Manville filters used in phase 2 gave mean total coliform counts equivalent to Millipore and Sartorius filters. The plaquing phenomenon was not observed in the preliminary field study, nor was it a problem in phase 3 where autoclaved Johns-Manville filters were used. In both the phase 1 and phase 3 studies, which used natural water samples,

Table 6. Recovery of faecal coliforms from Humber River water samples using Johns-Manville, Sartorius and Millipore membrane filters.

	Johns-Manville	Sartorius	Millipore
No. Comparisons	70	70	70
Mean ($\bar{x}$)	30.1	26.7	26.0
Standard Deviation	12.39	10.15	10.40

Analysis of Variance (ANOVA)

Source	SS	df	MS	F ratio[a]
Within	25150.87	207	121.50	
				2.75
Between	668.41	2	334.20	

[a] F .05∞ = 3.00

the mean coliform counts on the Johns-Manville filters were statistically greater than on Millipore and Sartorius filters. The autoclaved Johns-Manville filters however, were noticably more brittle and less flexible than the ethylene oxide sterilized filters. We also observed distortion of the faecal coliform colonies on autoclaved filters.

An additional undesirable features of the Johns-Manville and Millipore filters was the inhibition of growth by grid markings. Colonies growing near the grid lines developed flat edges, conforming to the restrictions imposed by the lines. Colonies which straddled the lines were split. Interestingly, grid line interference was more pronounced with total coliform than with the faecal coliform colonies.

We also noted, as did Dutka et al (2), that the Sartorius filters had irregular hydrophobic areas which became evident when the filters were wetted. These areas of reduced permeability likely contributed to the decreased bacterial recovery we observed with Sartorius filters. This was brought to the attention of the Sartorius representative about six months ago and we have yet to hear a satisfactory explanation of why this has occurred.

We realize that our culture media, M-Endo MF broth with agar added and M-FC broth base with agar added, are not recognized as standard solid media for membrane filtration by Standard Methods. LES Endo agar is the only solid medium accepted for the direct recovery of coliforms from water. However, we have conducted a comparison of coliform recovery on M-Endo MF broth with agar and LES Endo agar by membrane filtration of 101 natural water samples (unpublished). Statistical analysis indicated that there was no significant difference between coliform recovery on these two media.

The conflicting conclusions of our investigation and of other similar studies comparing membrane filters need to be resolved. Experimental designs including source of the test organism and statistical evaluations, must be standardized so that some logical conclusion regarding membrane filter performance can be made.

ACKNOWLEDGEMENTS

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QUESTION AND ANSWER SESSION

Geldreich: You mentioned that the use of the agar preparation of M-Endo and M-FC is a non-standard method. I would like to say that in the next edition of Standard Methods we have become so concerned about this problem, that I have written a paragraph saying that that you can certainly use it with 1.5% agar, and it will be a standard method. In fact, when I go out and do laboratory evaluations I certainly recommend it in my report.

Brodsky: We ran into problems, getting Endo base media from various companies. We were trying to find alternate procedures and this is how we got into it.

Brezenski: Could you please summarize. For example you said for EC positive organisms that Johns-Manville was equivalent to Millipore. Would you go over this? I think this is the crux of the issue, and I am a little confused because someplace where the line goes equivalent from here to here, it's not equivalent from here back again.

Brodsky: I must admit that I was confused too. As you realize the study was divided into 3 phases. Perhaps I should leave phase 1 out completely, because I really don't want to base a judgement on such a loosely controlled study. Let me do the 2nd and 3rd phases which are more complete and more rigidly controlled. In phase 2 we selected EC positive cultures, but for the sake of argument we will call them fecal coliform cultures, and then we compared their recovery, that is EC positive cultures on M-Endo media and on M-FC media in parallel, using the three same brands of filters. We did 5 replicates for each filtration. We determined that Johns-Manville and Millipore and Sartorius were equivalent when used with M-Endo MF medium for these EC cultures. When we used M-FC medium, obviously selected for fecal coliforms, we found that Johns-Manville was superior to Sartorius; however, Johns-Manville was equivalent to Millipore, and Millipore was equivalent to Sartorius. Now, there's some logic if you try and think if A=B, and B=C, then A must = C. Think of it this way, if A is 40, and B is 35, and C is 30. The difference between 40 and 35 is not significant. The difference between 35 and 40 is not significant, but the difference between 30 and 40 may be significant. It is confusing but that is the best explanation I can give you as to why this natural logic doesn't apply.

Then in phase 3 we said "fine." Let's see what happens now if we use natural cultures or a natural source of water, rather than using a laboratory culture in which we have given it the best possible condition to grow and allow them to overcome any possible inhibition. We were hoping to find some sort of parallel. Perhaps I shouldn't say that, because that isn't fair scientific judgement. What happened was for total coliforms from a natural polluted sample such as the Humber River, (I think anyone from the Toronto area can vouch for the fact that the Humber River is a polluted water source) we found that Johns-Manville was superior to Sartorius for total coliforms. If you recall in the first part we said that they were equivalent for total coliforms using the laboratory cultures. Johns-Manville and Millipore were equivalent and Millipore and Sartorius were equivalent for total coliforms. When we looked at fecal coliforms we didn't find any difference at all between the three brands of filters for recovering fecal coliforms from a natural polluted source. We performed two different studies; one used laboratory cultures and one used natural samples. We got two different results. We have to resolve this problem. The question that I'm asking is where do you get your source of culture? Obviously the source of culture is going to have a tremendous influence on conclusions.

COMPARISON OF MEMBRANE FILTERS IN RECOVERY OF NATURALLY INJURED COLIFORMS

by

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ABSTRACT

Raw sewage and cultures of E. coli were exposed to natural stream conditions in test chambers for 24 hours then recovered and enumerated with the M-FC test with and without enrichment and with different brand filters.

ANOVA, F and t statistics showed that the Gelman filters had only a slight advantage in recovery over Millipore filters. Both brands recovered significantly better than the Nuclepore. Enrichment gave significantly better recovery with variation by days. Pure cultures were more sensitive to test and filter variations than were the sewage cultures. The major conclusion was that Nuclepore membranes should not be used for coliform analyses in water.

INTRODUCTION

Beginning with Dr. William G. Walter's initial investigation (1964) into the relative bacteriological quality of water produced by adjacent open and closed watersheds, numerous studies of water quality in high mountain watersheds have been carried out in our laboratory using Millipore membrane filters exclusively. Presswood and Brown's article in 1973 (6) along with papers and discussions at the 1974 American Society for Microbiology meetings in Chicago, indicating that Millipore filters might be yielding erroneously low counts, caused some concern about the data collected over the last 10 years. With recent publications (2, 4, 7) adding conflicting data and interpretations to the issue, it seemed wise to follow the advice of Geldreich et al (3) and compare the performance of different brands of membranes in our laboratory.

MATERIALS AND METHODS

The preliminary experiments reported here were performed with suspensions of **Escherichia coli** C320 MP 25, isolated from water in our laboratory and with raw sewage. Procedures followed Standard Methods (1).

Aliquots of 24 hour cultures of E. coli were washed twice with gelatin phosphate buffer and dilutions yielding 10^5 to 10^6 cells per ml were placed in chilled river water, taken immediately to the stream site, and submersed in the flowing river. A 1 ml sample was taken from the chamber, placed in 9 ml gelatin phosphate buffer, iced and transported back to the lab where dilutions were plated on TSY agar (35 C) to yield a 0 time count. After 24 hours of exposure to the natural aquatic environment to allow injury to occur, a 1 ml sample of the contents of the chamber was transported to the lab in 9 ml of iced gelatin phosphate buffer. One ml of this cell suspension was placed in 9 ml of Trypticase soy broth + 0.5% glucose + 0.3% yeast extract and incubated at room temperature for 2 hours before filtering (enriched). Another 1 ml was taken from the buffer suspension, diluted, and filtered immediately (non-enriched). M-Endo MF medium (35 C) was used with the E. coli experiments. (mEndoMF-E. coli).

The sewage experiments were performed in an identical manner except that undiluted raw sewage was placed in the membrane filter chambers and

M-FC fecal coliform medium was used and incubated at 44.5 C (M-FC-sewage).

Colonies were counted with the aid of a binocular microscope (7X) and reflected light. Counts were statistically analyzed with regression analysis and analysis of variance utilizing the classifications: filter, day and enrichment.

RESULTS AND DISCUSSION

Results from three M-FC-sewage runs with 10 replicates with each brand of filter (Millipore, Gelman and Nuclepore) on non-enriched and enriched samples along with four identical mEndoMF-E. coli runs are reported in this paper.

Counts from the 10 replicate plates for each filter brand generally followed the Poisson distribution. The mean counts on Gelman filters were slightly higher than those on Millipore filters. Both Gelman and Millipore filter counts always exceeded counts on Nuclepore filters.

All counts were transformed using square roots and analyzed with a computer program for ANOVA. Both F and t statistics were computed for various interactions of means by using the Day-enriched-filter interaction mean square as the error term. Use of an interaction mean square as the error term is common in randomized block designs. Here one would consider a day as a block.

Day and enrichment differences were found to be significant at the P = 0.005 level for both the M-FC-sewage and the mEndoMF-E. coli situations. Enrichment effect varied significantly with different days (i.e. interaction) for both mEndoMF-E. coli (0.005) and M-FC sewage (0.05). Day by filter interactions and enrichment by filter interactions were not significant for M-FC-sewage but were significant (0.005 and 0.05 respectively) for mEndoMF-E. coli. This would seem to indicate a greater injury effect for the washed E. coli cells than for the raw sewage coliforms. The in-stream conditions varied during the course of the experiments resulting in changing degrees of injury which are reflected in the above statistics.

Differences among the 3 filters (all treatments grouped) were significant at the 0.01 level for M-FC-sewage and at the 0.005 level for mEndoMF-E. coli. Analysis of all Millipore counts versus all Gelman counts showed no statistical differences in either M-FC-sewage (0.2) or mEndoMF-E. coli (0.5) trials while differences between Millipore versus Nuclepore counts and Gelman versus Nuclepore counts were significant. These differences were much larger for the mEndoMF-E. coli situation (P = 0.001) than for the M-FC-sewage differences (P = 0.01 to 0.005) again suggesting a greater degree of injury for the E. coli compared to sewage coliforms, and subsequently, some kind of injury - Nuclepore filter interaction. If enrichment had overcome this inhibitory effect with Nuclepore filters, one would expect Nuclepore versus enrichment t values to be higher than those of Millipore and Gelman versus enrichment. This was not the case as shown by mEndoMF-E. coli t values of 49 and 54 for Millipore and Gelman versus enrichment effect and a t value of 41 for Nuclepore versus enrichment effect. Although the t values were smaller (3.9, 4.7, 3.2) this interpretation is corroborated by the results of the M-FC-sewage experiments.

A breakdown of filter comparisons into non-enriched and enriched trials showed Millipore versus Gelman differences to be insignificant for M-FC-sewage (0.5, 0.4) but were barely significant (0.05, 0.10) for mEndoMF-E. coli results. Millipore and Gelman were significantly different from Nuclepore at the 0.005 level in the mEndoMF-E. coli situation and from 0.05 to 0.005 in the case of the M-FC-sewage.

The practical significance of the differences between filter counts should be examined with an understanding of day to day changes in the natural environment and of errors and variation inherent in membrane filter techniques. For example, assuming that a set of replicate counts follows the Poisson distribution, the square root of the mean will give a reasonable estimate of the standard deviation one can expect between individual plate counts. Thus, for the mean of 52.6 observed for the M-FC-sewage Millipore counts, a standard deviation of ±6.7 would be expected. The actual difference between Millipore and Gelman means was observed to be 7.7 which is about equal to the expected variation among Millipore counts.

For all practical purposes, these data indicate that the bias towards higher counts obtained with Gelman as compared to Millipore filters is small. The difference between Gelman and Nuclepore means was 33.9, much larger than the expected standard deviation of 6.7. Thus, the error when using Nuclepore filters would be considerable.

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QUESTION AND ANSWER SESSION

Geldreich: Dave I think the reason you see so much difference between the Nuclepore and the other two is that the Nuclepore is not really the same material. As we tried to say this morning, if that membrane were to be used, you would have to redesign a whole family of media for it.

Stuart: I remember you saying that, and the reason that we used it was we don't use it for bacterial counts but when we are using algae in our chambers, we use a Nuclepore sidewall and it ended up being a pretty nice control.

EFFICIENCY OF COLIFORM RECOVERY USING TWO BRANDS OF MEMBRANE FILTERS

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ABSTRACT

The comparative study of Gelman and Millipore membrane filters by Presswood and Brown (5) prompted the evaluation of the two brands of membrane filters employing routine samples. The study included a total of 100 samples from a variety of non-chlorinated aquatic sources. Gelman filters averaged 2.5 times greater recovery of fecal coliforms than did Millipore filters. A comparative study, with fewer samples, was also made utilizing total coliform analyses. Data indicate that total coliform recovery is similar with the two brands. For verification as fecal coliforms, some typical blue colonies were subcultured from both filter brands to confirmatory media. Different lots of each brand of filters were used.

INTRODUCTION

Various brands of membrane filters have been under close scrutiny by several investigators. A disparity in the ability of different brands of membrane filters to support the growth of coliform bacteria from both natural and stock sources was found. Using a typical strain of fecal coliform, Levin et al. (4) observed that Gelman filters exhibited a much less adverse effect on the microorganism than did Millipore and Oxoid filters. Presswood and Brown (5), utilizing pure strains, found that Gelman filters recovered 2.3 times more fecal coliforms than did Millipore filters. Comparative analyses of river water for fecal coliform bacteria gave results comparable to those for pure cultures. In the study, total coliform recovery was statistically higher with Gelman filters than with Millipore filters. In a field study employing Gelman, Millipore, and Sartorius membrane filters, Dutka et al. (2) observed that Gelman filters generally produced the highest counts. Hufham (3) found that Gelman filters demonstrated higher counts of a strain of typical fecal coliform at 44.5 C than did Millipore filters; however, both filter brands showed similar results at 35 C. Using natural samples with Gelman and Millipore filters, Shaeffer et al. (6) obtained higher total coliform counts with Gelman filters. The fecal coliform counts were similar with the two membrane filter brands.

Over the past few years, the membrane filter test has become an official method (1) and a valuable laboratory tool. However, with the mounting data of various investigators indicating membrane filter brand disparity in microorganism enumeration, doubts have been raised concerning the present accuracy of the test.

As an in-house quality control measure, prompted by the results of Presswood and Brown (5), a study was initiated to evaluate Gelman and Millipore membranes using routine samples from non-chlorinated aquatic sources.

MATERIALS AND METHODS

Sample Sources and Sampling.

Water samples were obtained from three types of sources: (i) aerobic lagoon (influent and effluent); (ii) river; and (iii) sewage treatment plant effluent. All samples from these sources were collected in autoclaved sterilized bottles iced enroute to the laboratory, and processed within 8 hours of collection time.

Procedure, Culture Media, and Reagents.

All procedures, media, and reagents used were in accordance with those described in **Standard Methods for the Examination of Water and Wastewater** (13th ed.) part 400.

Samples were taken from the same dilution bottle and filtered simultaneously through each brand of filter using a Millipore membrane filtering apparatus with a 3 place-Hydrosol manifold.

Membrane Filters.

Three lots each of two commercial brands of 0.45 μm porosity membrane filters were used in the study: Millipore HAWG 047S0 (Millipore Corp., Bedford, Mass.) sterilized with ethylene oxide by the manufacturer; and Gelman GN-6 (Gelman Instrument Co., Ann Arbor, Mich.) sterilized in an autoclave by the manufacturer.

Confirmation of Colonies.

Fecal coliform colonies from membranes of both brands were tested to establish the validity of counts. Ten blue colonies were picked at random from each of 10 randomly selected membranes. The confirmation study was carried out in two phases: (I) subculture of 10 colonies per membrane to EC broth incubated at 44.5 C for 24 hours and (II) subculture of 10 more colonies per membrane to tryptophane broth, MR-VP broth, and citrate agar.

RESULTS AND DISCUSSION

Fecal coliform colonies grown on Millipore filters appeared larger, smoother, and more mucoid than on Gelman filters. The fecal coliform colonies on Gelman filters, although generally higher in number than Millipore filters, appeared small and often dull. This observation was also noted by Presswood and Brown (5). When utilizing the M-FC test, it was found that Gelman filters appeared blue while Millipore filters appeared beige-yellow. These have also been the findings of other investigators (2, 5). There has been speculation that this phenomenon is due to a difference in pH and that this could possibly be responsible for the disparity in counts on the two filters (5).

Table 1 shows that during 100 test trials, colony counts on Gelman filters were almost consistently higher than Millipore filters. On 3 trials, Gelman filters were lower or equal to Millipore filters in fecal coliform count. In considering the overall data, Gelman filters recovered 2.5 times more fecal coliform bacteria when the same samples and identical processing methods were used.

TABLE 1. COMPARATIVE STUDY OF GELMAN AND MILLIPORE FILTERS FOR THE RECOVERY OF FECAL COLIFORMS.

Colonies Per Membrane		Ratio	Colonies Per Membrane		Ratio
Millipore	Gelman	G/M	Millipore	Gelman	G/M
23	58	2.52	29	49	1.69
10	44	4.40	23	59	2.56
10	20	2.00	6	35	5.83
14	44	3.14	4	20	5.00
9	28	3.11	7	32	4.57
17	36	2.12	31	36	1.16
33	59	1.79	13	28	2.15
18	42	2.33	32	44	1.38
12	20	1.67	26	36	1.38
18	42	2.33	11	36	3.27
12	2-	1.67	10	36	3.60
7	21	3.00	10	30	3.00
18	43	2.39	7	46	6.57
19	38	2.00	44	43	0.98
26	36	1.38	11	22	2.00

Table 1 cont'd.

Colonies Per Membrane		Ratio	Colonies Per Membrane		Ratio
Millipore	Gelman	G/M	Millipore	Gelman	G/M
9	35	3.89	22	39	1.77
19	49	2.58	36	52	1.44
11	26	2.36	16	39	2.44
14	38	2.71	4	23	5.75
49	39	0.80	38	49	1.29
27	44	1.63	9	33	3.67
42	42	1.00	12	36	3.00
16	25	1.56	12	25	2.08
23	42	1.83	13	44	3.38
19	59	3.10	31	44	1.42
7	22	3.14	13	28	2.15
19	30	1.58	23	59	2.56
12	30	2.50	20	37	1.85
23	39	1.70	13	36	2.77
29	46	1.57	32	58	1.81
30	40	1.33	16	56	3.50
35	44	1.26	42	61	1.45
39	49	1.26	23	44	1.91
32	51	1.59	16	49	3.06
18	31	1.72	38	49	1.29
26	46	1.77	11	55	5.00
15	31	2.07	10	49	4.90
16	26	1.62	8	37	4.62
8	45	5.62	6	35	5.83
13	56	4.31	8	27	3.38
11	44	4.00	7	29	4.14
9	22	2.44	16	42	2.62
41	53	1.29	7	24	3.43
15	24	1.60	11	20	1.82
16	39	2.44	18	39	2.17
20	32	1.60	18	30	1.67
25	30	1.20	19	23	1.21
23	31	1.35	20	36	1.80
25	46	1.84	5	21	4.20
34	55	1.62	23	29	1.26

Total count Millipore = 1896
Total count Gelman = 3821
Mean count Millipore = 19
Mean count Gelman = 38
No. of times Millipore recovered:
- Higher counts 2
- Lower counts 97

No. of times Gelman recovered:
- Higher counts 97
- Lower counts 2

Σ of G/M = 251.51
Mean ratio of G/M = 2.52

Table 2 indicates that total coliform recovery is similar with the two brands. The contrasting data of Tables 1 and 2 would indicate a possible elevated temperature-membrane inhibitory effect. This difference in recovery has been observed by other investigators (2, 3, 5). However, Presswood and Brown (5) found a statistically significant difference at both 35 C and 44.5 C. The comparative total coliform study is not as comprehensive as the fecal coliform study due to: (1) initial tests indicated the two membrane filter brands gave similar counts (2) the majority of samples processed in our laboratory are for fecal coliform determination. It is hoped that a more in-depth membrane comparative study with total coliform procedures will be a part of our quality control program in the near future.

Due to the higher fecal coliform counts on Gelman membranes, it was necessary to confirm typical colonies in order to eliminate the possibility of a high number of false positives on Gelman membranes. Table 3 indicates that the two membrane filter brands gave similar confirmatory results in both phase I and II.

TABLE 2. COMPARATIVE STUDY OF GELMAN AND MILLIPORE FILTERS FOR THE RECOVERY OF TOTAL COLIFORMS.

Colonies Per Membrane		
Colonies Millipore	Membrane Gelman	Ratio G/M
50	54	1.08
25	24	0.96
25	24	0.96
25	27	1.08
26	28	1.08
44	38	0.86
33	24	0.73
21	17	0.81
29	35	1.21
19	24	1.26
19	26	1.37
42	30	0.71
17	24	1.41
32	25	0.78
19	29	1.53

Total count Millipore = 426
Total count Gelman = 429
Mean count Millipore = 28
Mean count Gelman = 29
No. of times Millipore recovered:
    Higher counts = 7
    Lower counts = 8
No. of times Gelman recovered:
    Higher counts = 8
    Lower counts = 7
Σ of G/M = 15.83
Mean Ratio of G/M = 1.06

TABLE 3. VERIFICATION OF TYPICAL BLUE COLONIES ON MILLIPORE AND GELMAN MEMBRANES

	Filter Brand	No. Colonies* Picked	No. EC Positive
I	Millipore	100	92
	Gelman	100	90

	Filter Brand	No. Colonies* Picked	IMViC Pattern	No. of Cultures
II	Millipore	100	+ + - -	97
				1
			- + - -	2
	Gelman	100	- - + -	95
				1
			- - + +	4

* 10 typical blue colonies were picked at random from 10 randomly selected membranes.

In order for a microbiologist to report accurate data, he must have efficient tools. Hopefully, there will be a standardization and a centralized routine evaluation of membrane filter brands. The varying efficiencies of membrane filter brands cannot be allowed to continue.

SUMMARY

(1) A comparative study has been conducted with two brands of membrane filters.

(2) The data indicated that Gelman filters recovered 2.5 times more fecal coliforms than Millipore filters.

(3) Recovery of total coliform bacteria was similar with Gelman and Millipore filters.

(4) There is a need to improve the quality control of membrane filters for use in water microbiology.

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The authors wish to express their appreciation to H. Bunczewski of the Bio-Analysis Group, U.S. Environmental Protection Agency, Region VII Laboratory, for media-reagent preparation and filtration of various samples. A special note of thanks to S. Finley of the same laboratory for her clerical assistance.

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QUESTION AND ANSWER SESSION

Sladek: The only comment I have is with respect to your statement on good lots and bad lots. I don't believe we came to exactly that conclusion. One of the lots that Mr. Harris studied was about 5 years old and we had manufactured it at a time before we had begun to quality control those filters with respect to the fecal coliform test.

Harris: Well how do you explain that the two good lots gave lower recovery compared to the Gelman lots?

Sladek: I think without being able to inspect the numbers and the experimental conditions, it is not really worth discussing.

Dazio: I would like to ask you or anybody else in the audience, if you could tell me what basic difference there is between different brands, let's say Gelman and Millipore, which could account for the differences for the recovery of fecal coliforms. Does anyone know? I think we should being asking some basic questions, and analyse and perhaps chemically determine what differences exist in the composition of membranes which could account for differences in recovery.

Harris: Well, there is one possibility. The Gelman membrane has phosphate in it whereas Millipore does not. Millipore filters are supposedly almost completely inert. So it could be a possibility that the phosphate in Gelman membranes acts as a nutrient source for the fecal coliform organism and therefore tends to enhance the growth of the fecal coliform organism on the membrane.

Question from the Floor: Somebody, this afternoon, said that the phosphate buffer was very delaterious to fecal coliform growth.

Grasso: I want to ask the gentleman from the University of Florida, "Will you be here tomorrow?" I think that Dr. Litsky put me in my place and said to hold my information until I present my paper. So, without letting it out of the bag today, I think I could probably give you some encouraging results on what is the effect of different brands of filters tomorrow in our paper. Thank you.

Harris: We have some comments that ethylene-oxide leaves a toxic residue but according to the presentation by Millipore Corporation this morning they can't find it.

Brodsky: Not to put down the individual companies producing membrane filters, but I am curious to know where you got your membrane filters? Were they, pardon me, a donation from the company or were they purchased from stock?

Harris: They were purchased from stock.

Brodsky: From a stock, both brands?

Harris: Right, both brands.

Brodsky: O.K. Because I think that is one aspect of the investigation that really should be settled.

Harris: We did not go directly to Gelman to get the filters or directly to Millipore, they were from the clearing house.

Rusnell: I just wanted to know if the samples were chlorinated or unchlorinated.

Harris: We took this into consideration before starting our study, and we decided that it wouldn't be wise to use chlorinated sources.

Mack: I was wondering if anybody had taken a look at the coliform organism under the electron microscope, because we are talking about filter sizes and all the scientific work that goes with this and nobody has come up with the fact that many of these organisms are terribly large and some are quite small. Some of them have capsule-like material and others don't, and this would have a great deal to do with whether or not they are retained.

Lane: You said that the Gelman membranes were autoclave-sterilized and Millipore were ethylene-oxide sterilized. How can you compare one membrane that is sterilized by one method with a membrane that is sterilized by an entirely different method? Shouldn't they both be sterilized the same way?

Harris: According to the manufacturers they are supposed to give equal results. There are not supposed to be inhibitory effects from ethylene oxide or autoclave sterilization. Millipore says ethylene oxide is nontoxic so we used their filters and compared to autoclaved filters.

Lane: I can't visualize that heating a membrane to the temperature of 121° C for 20 minutes or a half an hour is not going to do something to the pore size of the membrane whereas ethylene-oxide might be nontoxic.

Harris: But the fact remains one gets higher counts than the other.

Presswood: Ours was a comparison of filters similar to Mr. Harris' and we also used autoclave and ethylene oxide membranes. The reason we did this was that this is the way the manufacturer sells them. I think Millipore does sell autoclaved filters but as Mr. Harris said the ethylene-oxide is suppose to give comparable results. Also some of the Millipore filters become distorted if you do autoclave them. You can see by the grid lines, that the filters are distorted.

Question from the Floor: What about Nuclepore?

Furman: The Nuclepore membranes do not grow colonies with media that you use, so I would like to publicly tell you not to use Nuclepore for that purpose and with these media. We have never made the claim that the polycarbonate would work for water analyses. If you have some research needs for polycarbonate and you can use some wrinkles, add surfactants to the media to improve growth. But for the routine use we do not recommend Nuclepore for microbiological purposes. Thank you.

COMPARISON OF MEMBRANE FILTER BRANDS FOR RECOVERY OF THE COLIFORM GROUP

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ABSTRACT

Recoveries of pure cultures of **E. coli, K. pneumoniae** and **E. cloacae** and fecal coliforms from natural waters were compared using the M-FC test and different lots and brands of membrane filters. MPN recoveries were used as reference values for measuring accuracy.

Except for Nuclepore, the brand of membrane filters did not significantly affect recoveries of pure cultures or natural source fecal coliforms. Variability increased with natural water samples. The variability (precision) of results from lot to lot within a brand and from culture to culture within an MF brand precluded any generalization about acceptance of one brand over another.

INTRODUCTION

Our interest in the recovery efficiencies of membrane filters originated a few years ago when we began an epidemiological/microbiological study of the relationship between pollution levels and the incidence of disease at marine bathing beaches. It resulted from the necessity to evaluate standard membrane filter (MF) methods in marine waters prior to their possible use in the study. The evaluation of the M-FC test revealed that it measured much lower fecal coliform densities than the EC most probable number (MPN) test. This in turn led to a further investigation of various membrane filter brands in order to determine if the filters themselves affected the accuracy and precision of the assay method. For the purpose of this presentation, I want to define those two terms as we use them.

The accuracy of a method for an organism or a group of organisms, which may be defined as the ability to detect the "true density" of bacteria in a given volume of a water sample, ideally should be determined using natural samples. However, since "true density" is, in fact, unknown in natural samples, the accuracy can be determined only relative to some "standard" or "reference" method. Secondly, when a group of organisms is being enumerated i.e., coliforms or fecal coliforms, the relative accuracy may vary from location to location depending on the particular distribution of the component biotypes in the particular sample.

The precision of a method, which may be defined as the degree of dispersion of obtained values around a mean estimate, is equally as important as its accuracy or recovery efficiency. This is so because of the practice of determining bacterial density values with a single filter which is a common practice. Thus, a lack of precision caused by the filter would affect the ability of a method to detect differences between bacterial densities of two water samples.

With these two parameters in mind, the following experimental methodology was used to determine which type of filter would be most efficient for our purposes. I would like to state that all of these were done with surface waters in mind, particularly bathing beach surface waters, and I would not like to expand this to other types of water samples.

MATERIALS AND METHODS

Media Dehydrated M-FC broth, M-Endo broth, nutrient agar, Trypticase soy broth, EC broth, lactose broth and brilliant green lactose bile broth were obtained from Difco and prepared for use according to the directions of the manufacturer.

Test Organisms Strains of E. coli, **Klebsiella pneumoniae** and **Enterobacter cloacae** were maintained on nutrient agar slants. The **Klebsiella** strains were able to produce gas in EC broth incubated at 44.5 C.

Filters Membrane filters from the following manufacturers were obtained from commercial sources: Millipore (M), Gelman (G), Sartorius (S), Schleicher and Schuell (s/s) and Nuclepore (N). All of the membranes were packaged sterile with the exception of the Nuclepore and Schleicher and Schuell membranes, which were sterilized by autoclaving for 15 minutes at 121 C. All membranes, with the exception of Nuclepore, were gridded and had an average pore size of 0.45 microns. Nuclepore membranes were ungridded and had a 0.4 micron pore size.

Natural Samples Natural samples were obtained from three locations in the Rhode Island area. They were: (1) Wickford Harbor, a salt water cove which receives mainly septic tank overflows, and this would be human waste of which the major component was **E. coli**. (2) the Saugatucket River, which receives mainly industrial effluent from a textile finishing plant and these effluents contained **Klebsiella** species. (3) the Pawcatuck Estuary whose bacterial pollution comes from domestic sources, as well as industrial effluents. The samples usually arrived at the laboratory within three hours of collection and they were immediately assayed.

Test Cell Suspensions Eighteen to twenty hours before each experiment, a loopful of the test strain was transferred to Trypticase soy broth. After 18 hours incubation at 35 C, the culture was diluted in sterile, phosphate-buffered saline (pH 7.2) to a density of between 20 and 60 organisms per ml.

Membrane Filtration One ml of the test cell suspension, or an appropriate volume of a natural sample, was passed through each filter after being mixed with 20 ml of buffer. The filter was then rolled onto a broth saturated pad or the agar surface of a randomly chosen plate. The M-FC plates were incubated in "whirl-pak" bags in a 44.5 C water bath. The M-Endo plates and membrane nutrient agar plates were incubated in an inverted position in a 35 C incubator. All membrane filter brands were tested in triplicate on M-Endo broth, M-FC broth and nutrient agar media.

Control Procedure Spread plates used to determine the density of the test cell suspensions, were prepared by pipetting 0.2 ml of the test cell suspension onto each of five plates. The suspensions were spread over the surface of the nutrient agar medium with a sterile glass rod and allowed to dry, after which the plates were incubated in an inverted position at 35 C.

The MPN procedures were carried out as described in Standard Methods for the Examination of Water and Wastewater (1).

Statistical Analysis Tukey's Studentized Range Procedure for comparing several means was used for the statistical analysis of the data (2). The dispersion about the mean was described as the coefficient of variation.

RESULTS AND DISCUSSION

The accuracy of the M-FC procedure for fecal coliforms as it is affected by the brand of membrane filter used, was determined by comparing the densities in **Klebsiella** and **E. coli** test suspensions obtained by the MF procedure with those from nutrient agar spread plates. It was assumed that the density, as determined from nutrient agar spread plates, would provide the best estimate of the number of bacteria actually present in a given test cell suspension. The effect of five membrane filter brands on the accuracy of the M-FC procedure with four fecal coliform strains is shown in Table 1. The percent recoveries for the **E. coli** strains ranged from 16 to 75%, and for the **Klebsiella** strains they ranged from 14 to 94%. Although the filter brands are ranked according to their mean recovery values, most of the observed differences were not statistically significant as shown by the underscoring of mean recovery values of each organism. The underscoring indicates that any two means, not underlined by the same line, are significantly different at the 95% confidence level. Statistically significant differences appeared more frequently with the **Klebsiella** strains than with the **E. coli** strains. Of the brands tested, the Gelman product appeared to provide the most, and Nuclepore the least accurate density estimates with the four fecal coliform strains tested. However, none of the filter brands was consistently accurate. This appeared to depend more on the basic method relative to the strain being tested rather than the filter brand.

TABLE 1. RECOVERY OF **E. COLI** AND **K. PNEUMONIAE** ON THE M-FC MEDIUM WITH VARIOUS MEMBRANE FILTER BRANDS

E. coli	no. 3					
	Filter type (1)	N	M	s/s	S	G
	Mean (2)	14	34	38	41	46
	% Recovery	16	39	43	47	52
	S.T. (3)		———	———	———	———
E. coli	no. 5					
	Filter type	N	s/s	S	M	G
	Mean	50	57	71	77	98
	% Recovery	38	44	55	59	75
S.T.	S.T.	———	——— ———	——— ———	 ——— ———	 ———
K. pneumoniae no. 450						
	Filter type	M	S	s/s	N	G
	Mean	21	29	31	47	75
	% Recovery	14	19	20	30	48
	S.T.	———	———	———		
K. pneumoniae no. 444						
	Filter type	N	s/s	S	M	G
	Mean	22	66	86	99	118
	% Recovery	18	53	68	80	94
	S.T.		———	——— ———	 ——— ———	 ———

1. Gelman (G), Millipore (M), Nuclepore (N), Sartorius (S) and Schleicher and Schuell (s/s)
2. Relative to bacterial density on nutrient agar spread plate incubated at 35C.
3. S.T. = Statistical Test: Tukey's Studentized Range Procedure; all means not underscored by the same line differ significantly at the P = 0.05 level.

The effect of membrane filter brand on the precision of the M-FC test is shown in Table 2. The filters are ranked according to the magnitude of dispersion around the mean. The precision of the Sartorius filters was consistently better (a lower coefficient of variation) than the other brands, and the Nuclepore and S & S filters were consistently poor. Unlike the accuracy, the precision appears to be filter brand dependent rather than strain dependent.

The recovery of fecal coliforms from natural samples is shown in Table 3. The trend, with regard to filter efficiency, appears to be similar to that found with pure culture suspensions. However, there were fewer statistically significant differences between filter brands with natural sample suspensions. These differences were probably due to the greater filter variability of all brands (see Table 4). This variability would, in fact, cause the statistical test used to be less sensitive for detecting recovery differences between filter brands.

Table 4 indicates that the precision of the M-FC test was much lower with all brands of filter when compared with the pure culture data. It can also be noted that the rank position of the filter brands was not similar to those obtained with pure cultures. This is probably indicative of the heterogeneous nature of the fecal coliform group.

TABLE 2. PRECISION OF MEMBRANE FILTER BRANDS ON M-FC MEDIUM

E. coli no. 3

Filter type (1)	N	s/s	G	M	S
C.V. (2)	43	24	17	12	5

E. coli no. 5

Filter type	s/s	N	G	M	S
C.V.	18	12	12	6	3

K. pneumoniae no. 450

Filter type	N	M	s/s	G	S
C.V.	19	14	10	7	4

K. pneumoniae no. 444

Filter type	N	s/s	M	G	S
C.V.	45	18	14	3	2

1. See footnote 1, table 1.

2. C.V. = Coefficient of Variation; (standard deviation/arithmetic mean) x 100

TABLE 3. FECAL COLIFORM RECOVERIES FROM NATURAL SAMPLES WITH VARIOUS MEMBRANE FILTER BRANDS USING THE M-FC TEST.

Wickford Harbor

Filter type (1)	N	s/s	S	M	G
Mean (2)	8.3	12	17.7	20.3	21.3
% Recovery	12	17	25	29	30
S.T. (3)					

Saugatucket R.

Filter type	N	s/s	M	G	S
Mean	4	5	6	8	12
% Recovery	33	38	46	62	92
S.T.					

Pawcatuck Estuary

Filter type	N	s/s	M	S	G
Mean	1	4.7	5.2	12	16
% Recovery	7	32	36	82	109

1. See footnotes 1 and 3, table 1.

2. Relative to E.C. MPN estimate

TABLE 4. PRECISION OF MEMBRANE FILTER BRANDS ON M-FC MEDIUM

Wickford Harbor						
	Filter type (1)	s/s	G	S	N	M
	C.V. (2)	35.8	35.7	25	18.7	7.5
Saugatucket R.						
	Filter type	N	M	G	s/s	S
	C.V.	78	45	44	20	17
Pawcatuck Estuary						
	Filter type	N	M	S	s/s	G
	C.V.	100	40	25	22	19

1. See footnote 1, table 1.

2. See footnote 2, table 2.

The effect the filter brand has on the recovery of total coliforms by the M-Endo procedure was also examined. Table 5 shows that, with pure cultures of E. coli, **Klebsiella**, and **Enterobacter cloacae**, recovery between brands was neither appreciable nor statistically significant. The one exception was the Nuclepore brand, which was consistently poor in its ability to recovery coliforms. Relative to spread plates on nutrient agar (NA), all of the filter brands, with the exception of Nuclepore, were reasonably accurate when using **Klebsiella** or **Enterobacter** as the test strain. The recoveries with E. coli no. 3 were rather poor. In order to determine if this might be due to inhibitors present in M-Endo medium, the mean recoveries on M-Endo medium were compared to those of membranes also placed on nutrient agar. As can be seen in Table 6, the relatively poor recoveries of E. coli no. 3 on M-Endo were also obtained on NA, suggesting a general filter effect or a nutrient deficiency for this particular organism.

The relative accuracy of the various filter brands, when examined using natural samples was essentially the same as noted above. The recovery relative to the MPN procedure was excellent in two of the three samples examined. However, the fact that the MPN procedure was used to estimate the "true" coliform density does not allow substantive conclusions to be drawn about these data in this regard. See Table 7.

The variability, from lot to lot, of some filter brands would preclude broad generalizations about the acceptability of one brand over another except for those brands actually tested under the same conditions. This point is illustrated in Table 8. In two out of the three brands tested, there were statistically different mean recovery values between lots. Thus, it would appear that with some membrane filter brands, the recovery efficiencies can not be predicted from lot to lot. And you will notice that this is a different E. coli strain than we used in the other three experiments. What happened is that we had lost this strain, but last week we found a lyophilized culture of it. So, we ran it through the same test procedure and, if you will put on the last slide, you will see the strain to strain variabilities causing differences with the Gelman filters.

SUMMARY

The following conclusions can be made:

1. The use of pure cultures to detect differences in accuracy in the M-FC procedure between membrane filter brands is more sensitive than using natural samples. However, there is a strain to strain variation in the way in which pure cultures react to each filter brand and

TABLE 5. RECOVERY OF E. COLI, K. PNEUMONIAE AND E. CLOACAE ON M-ENDO MEDIUM WITH VARIOUS MEMBRANE FILTER BRANDS

E. coli no. 3

Filter type (1)	N	G	s/s	M	S
Mean (1)	32	58	59	61	68
% Recovery	36	66	67	69	77
S.T. (1)					

K. pneumoniae no. 444

Filter type	N	G	M	s/s	S
Mean	68	107	109	112	120
% Recovery	51	86	88	90	105
S.T.					

K. pneumoniae no. 450

Filter type	N	s/s	S	G	M
Mean	8	14	15	15	20
% Recovery	44	78	83	83	111
S.T.					

E. cloacae no. 491

Filter type	N	s/s	S	G	M
Mean	11	23	24	243	26
% Recovery	58	121	126	128	137

1. See footnotes 1, 2 and 3, table 1.

TABLE 6. COMPARISON OF E. COLI RECOVERIES ON M-ENDO AND NUTRIENT AGAR WITH VARIOUS MEMBRANE FILTER BRANDS

E. coli no. 3

	% Mean Recovery Value (1)				
Filter type (1)	N	G	s/s	M	S
mEndo	36	66	67	69	77
Nutrient Agar	52	70	68	69	75

1. See footnotes 1 and 2, table 1.

TABLE 7. TOTAL COLIFORM RECOVERIES FROM NATURAL SAMPLES WITH VARIOUS MEMBRANE FILTER BRANDS ON M-ENDO MEDIUM

Wickford Harbor

Filter type (1)	N	s/s	G	S	M
Mean	9	33	37	38	39
% Recovery (2)	26	94	106	109	111
S.T. (1)					

Saugatucket R.

Filter type	N	G	S	s/s	M
Mean	27	56	67	71	83
% Recovery	15	32	38	41	47
S.T.					

Pawcatuck Estuary

Filter type	N	s/s	G	S	M
Mean	8	31	33	36	37
% Recovery	22	86	92	100	103

1. See footnotes 1 and 3, table 1.
2. Relative to completed total coliform MPN estimate.

this may influence the choice of a "best" membrane filter. Furthermore, pure culture may not truly mimic the physiological state of organisms in natural samples.

2. The precision of the M-FC test is influenced by the filter brand, and this is a rather consistent characteristic from strain to strain with pure cultures.
3. Differences in precision due to membrane filter brand have a tendency to be lost when natural samples are used as the test inoculum in the M-FC test. This is assumed to be caused by the heterogeneity of the fecal coliform group and the physiological state of the organisms.
4. The relative accuracy of the various brands of membrane filters was essentially the same whether pure cultures or natural samples were used. However, in the latter case the differences were masked due to the decreased precision when natural samples were used.
5. In general, acceptable total coliform recoveries were obtained by the M-Endo procedure with all the filter brands (except Nuclepore).
6. If pure cultures are used to compare various filter brands or lots within a filter brand, the choice of test organism is exceedingly important, since, with some strains, an overall membrane filter effect may mask true differences. If natural samples are used, a large enough number of filters must be examined to compensate for the decreased precision observed with this type of test suspension.
7. Three factors that must be considered in evaluating membrane filters are accuracy, precision, and lot to lot variability.

REFERENCES

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TABLE 8. RECOVERY OF E. COLI ON M-FC MEDIUM WITH DIFFERENT LOTS OF MEMBRANE FILTER BRANDS

Millipore
(E. coli no. 104)

Filter lot #	1295	NN	9396	6112	8752	9733
Mean	12.3	25.7	26.3	38.3	47.7	89.7
% Recovery (1)	10.4	21.7	22.3	33	40	76
S.T. (1)						

Millipore
(E. coli no. 104)

Filter lot #	3283	5000	9768	9733
Mean	6.3	16.7	17.7	62
% Recovery	6.3	16.7	17.7	62
S.T.				

Gelman
(E. coli no. 5)

Filter lot #	80714	80706	80730
Mean	70	75	76
% Recovery	73	78	79
S.T.			

Sartorius
(E. coli no. 104)

Filter lot #	10	30
Mean	65	27
% Recovery	34	14
S.T.		

1. See footnotes 2 and 3, table 1.

2. Lecture Notes in Applied Statistics, Joseph L. Ciminera, Villanova Univ. Press, 1956.

QUESTION AND ANSWER SESSION

Geldreich. What do you think of the possibility of using pure cultures in some of the evaluations of these products? We can't say, as you are saying, to use a natural sample, although I always like to use them in preference to pure cultures. What about the possibility of using a mixture of pure cultures in a lyophilized condition in which you can standardize and then use them as a way to come up with a test group which would allow us to look for sensitivity, as well as selectivity in suppressing some organisms. You think this is a possible way out of the problem?

Dufour. I don't really know. Dr. Hufham outlined this rather well in his presentation this morning. Any one culture has a certain part of the distribution, even if we called it pure culture, that is killed by the elevated temperature. I don't know whether putting in different types of organisms and using this as a test suspension will overcome that shortcoming.

Geldreich. One of the problems that we have here is that if you use an E. coli of a certain type, you are only checking it for one thing, and that is sensitivity to let's say E. coli. But, if you are trying to check it for suppression of other organisms, you would be missing that point.

Dufour. Yes. This is why we used samples from what we thought were three different environmental situations. But obviously, we couldn't show anything because of the great dispersion we found between filters.

Geldreich. We noted, and you may have too, for instance that Gelman membranes may at times have more background count of other organisms growing on them. This may occur because of some nutrient material present there.

Dufour. Again, this was not one of the problems that we ran into because of the way we chose our natural sample.

Presswood. I noticed that if you are using pure cultures, the source where you isolate the culture makes a difference. I have isolated some bacteria from raw sewage before it was chlorinated and those bacteria did not respond as well to the membrane filter technique as bacteria isolated from river water. Even after passing them through 2 or 3 passes of EC medium, or some other nutrient broth, they still did not respond as well to the membrane filter. Especially at 44.5C as E. coli, or what we call fecal coliform bacteria isolated from river water. I don't know why this is, but it happens.

Dufour. It was mentioned today, that all of our strains were not isolated using the M-FC procedure. They were isolated using a non-inhibitory medium and found to be fecal coliforms using EC broth. So, at least we didn't have that shortcoming.

Bordner. It just occurs to me that one of the plans we have in mind at MDQARL, Methods Development and Quality Assurance in Cincinnati, is to accept bids for a contract that will develop pure cultures from environmental samples and fecal samples. These can then be used as reference samples. It occurs to me that it might be appropriate to look at this type of culture which we hope to have lyophilized and made available to our laboratories and others later on for membrane filter and media evaluation.

A COMPARISON OF MEMBRANE FILTERS, CULTURE MEDIA, INCUBATION TEMPERATURES, POLLUTED WATER AND ESCHERICHIA COLI STRAINS IN THE FECAL COLIFORM TEST

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ABSTRACT

Twenty lots of membrane filters from 3 manufacturers produced variable results when the fecal coliform test was used with polluted water samples and **Escherichia coli** (**E. coli**). The growth of nineteen **E. coli** cultures varied with incubation temperatures of 35, 43, and 44.5 C, with membrane filter lots, and with culture media (Trypticase soy agar, M-FC, and violet red bile agar). Growth was better on violet red bile agar than on M-FC medium, which was not always due to the 44.5C incubation temperature. Some of the **E. coli** strains tested did not grow as well in poured agar plates as they did on membrane filters. Evaluation of the results indicated that the efficacy of the membrane filter for the fecal coliform test could be affected by, (1) the strain of **E. coli**, (2) the method utilized for growth (pour plate, pad and broth, pad and agar), and (3) the temperature inside the plate. Therefore, it is apparent that test methods must be standardized for these factors.

INTRODUCTION

The membrane filter method used for measuring fecal coliform bacteria in polluted water has been critically evaluated by several investigators. Poor recovery of fecal coliform or **E. coli** strains has been attributed to the variation of the quality of the components in the M-FC agar (10), to the differences in the brands and lots of the membrane filters (1,5,8,11), to the variation in the incubation temperatures (3), and to the type of water tested (1,7,9).

This study was undertaken to determine the efficiency of three brands of membrane filters to recover fecal coliforms and **E. coli** isolated from polluted water. Three incubation temperatures (35, 43, and 44.5 C) were studied to determine the effect of temperature on the growth of the bacteria. A non-inhibitory medium, Trypticase soy agar (TSA, BBL), was used to determine the actual counts of the **E. coli** cultures being compared for growth on M-FC and violet red bile agar (VRB, BBL). Additional studies were initiated to determine the cause of the erratic behavior of the membrane filters and the **E. coli** bacteria.

MATERIALS AND METHODS

The manufacturer's name and lot number of the membrane filters (with the prefix used as a test number in this study) were as follows:

Test No.	Millipore Lot No.	Test No.	Gelman Lot No.	Test No.	Sartorius Lot No.
M-1	95434-3	G-3	80730	S-1	713 649
M-3	93448-10	G-6	80822	S-2	753 649
M-5	11013-4	G-7	80901	S-3	773 284
M-6	95434-2			S-4	993 447
M-7	12732-6			S-5	993 711
M-8	12667-3			S-6	993 186
M-11	06602-8			S-7	013 870
M-12	06618-3			S-8	773 568
M-13	34798-4				

All of the above except M-11 and M-12 were pre-sterilized by manufacturer. These two lots were used as supplied (not sterile).

M-FC broth BBL lot 204625) with 0.01% rosolic acid (Difco lot 488535) added was used for tests with pads, and 1.5% agar added for pour plates. These two media were dissolved by heating to a boil (as was the VRB). The TSA was autoclaved at 121 C for 15 minutes. The agar media were cooled to 45 C before pouring plates.

Sterile demineralized water (pH 6.5) was used to prepare dilutions of the **E. coli** culture and as a dispersal solution (20 to 30 ml) during filtration of all samples. Thirty to 40 ml of this water was filtered after every 5 replicates as controls. A 1,000 μ1 Eppendorf, or a 5 to 10 ml Oxford pipet with sterile tips, was used to transfer 1 to 10 ml of diluted **E. coli** culture, or creek water, for filtration and agar pour plates. Membranes were placed on pads in sterile 48 x 8.5 mm Millipore or 12 x 50 mm Falcon plates. Sterile 15 x 100 mm plastic petri dishes were used for pour plates. The upper half of the Gelman magnetic filter funnel was placed under a germicidal lamp (G.E. G8T5) between filtrations. Plates were sealed inside Whirl-pak (Nasco) or Ziploc (Dow Chemical Co.) plastic bags prior to immersion in the water baths. After 24 hours incubation, typical blue M-FC or red (VRB) colonies were counted at 7X magnification with daylight fluorescent illumination. The 15 x 100 mm agar pour plates were counted using a Quebec colony counter. Plates with 20 to 100 colonies for the 48 x 8.5 mm plates and 30 to 300 for the 100 x 15 mm plates, were considered countable.

The nineteen **E. coli** cultures isolated from creek water were incubated at 44.5 C on M-FC medium. Biochemical reactions of the **E. coli** cultures tested produced acid-slope and acid-gas butt on triple sugar iron agar (TSI, BBL). The Minitek (Bioquest) test method gave the following results: positive for indole, methyl red, lactose, arabinose, lysine decarboxylase; negative for Voges-Proskauer, citrate, phenlalanine, inositol, malonate, H_2S.

Variations occured with ornithine decarboxylase and rhamnose respectively as follows: +, +, Nos. 2A, 3A, 9, 16, 16A, 16X, 16Z, 24; -, +, Nos. 1A, 5; -, -, Nos. 1, 2, 2Z, 3, 3X, 3Z; 4, +, -, Nos. 2B, 2X. Culture #4A gave results typical of **Klebsiella pneumoniae.**

Stock cultures were maintained at room temperature on TSA slants sealed with waxed corks. Growth cultures were transferred from stock plants to VRB agar plates, incubated at 35 C overnight, and a single colony suspended in sterile demineralized water (pH 6.5). One ml of the suspension was transferred to membrane filters, or to pour plates for subsequent tests.

The temperatures of the Hotpack Incubator #5528 (35 C), the GCA-Precision Scientific Co. Coliform Incubator Bath (44.5 C), and the Blue-M non-circulating waterbath (43 C) were continually monitored during the study. The waterbath temperatures and the temperatures of the corresponding immersed plates did not vary.

The different lots of Gelman, Millipore and Sartorius membrane filters were tested with 11 **E. coli** cultures. The membrane filters were placed either on corresponding pads saturated with M-FC medium or on TSA plates and incubated at 44.5 C (GCA-Coliform Bath). The same test cultures were also plated with TSA agar and incubated at 35 C. One ml of a suitable dilution of the culture was used for filtration and for poured plate counts. All tests were done in five replicates.

The results based on the average counts, are expressed as percent (%) recovery of **E. coli**. The pour plate counts obtained with TSA at 35 C were considered representative of the actual number of **E. coli** in the 1 ml volume tested. Membrane filters were placed on solid TSA agar for comparison with membrane filters placed on pads. A dilution of one strain of **E. coli** was tested with all membrane filters listed for that strain at one time.

RESULTS

Comparison of E. coli Recovery by M-FC Brand and Lot

In Table 1 the results are presented in 3 categories, A, B, and C. Category A is the percent recovery of the **E. coli** counts on membrane filters

TABLE 1 PERCENT RECOVERY OF E. COLI ON M-FC COMPARED WITH TSA AT 35 C AND 44.5 C.

Filter Lot No.*	E. coli Culture	A M-FC 44.5 TSA 44.5	B M-FC 44.5 TSA 35	C TSA 44.5 TSA 35	Filter Lot No.	E. coli Culture	A M-FC 44.5 TSA 44.5	B M-FC 44.5 TSA 35	C TSA 44.5 TSA 35
M-6	1	83	80	96	M-6	3A	95	59	62
G-3	1	84	109	131	G-3	3A	102	70	69
S-8	1	67	81	120	S-4	3A	104	60	57
S-3	1	87	82	94	G-6	3A	97	60	62
None**	1	52	58	111	S-6	3A	78	49	62
M-13	1A	0	0	113	M-13	3Z	81	74	91
G-3	1A	23	26	106	G-3	3Z	80	91	114
S-1	1A	0	0	73	S-3	3Z	44	47	107
M-3	1A	0	0	96	M-13	4	83	68	82
G-6	1A	22	17	83	G-3	4	71	72	101
S-4	1A	0	0	88	S-3	4	60	55	92
M-13	2	74	53	72	M-7	9	75	87	117
G-3	2	114	73	64	G-6	9	88	100	114
S-1	2	99	55	55	S-5	9	77	80	104
M-5	2A	81	62	77	G-3	9	78	81	104
G-6	2A	88	57	64	S-7	9	64	62	97
S-3	2A	74	52	70	M-8	16	82	66	81
G-3	2A	97	65	81	G-3	16	96	82	85
S-5	2A	90	58	65	S-6	16	71	66	93
M-13	3	55	52	95	G-6	16	71	65	92
G-3	3	72	66	92	S-8	16	75	64	84
S-1	3	64	53	84	M-12	24	133	78	59
					G-6	24	75	64	84
					S-7	24	92	73	79
					G-3	24	87	74	85
					S-1	24	95	70	74

* Millipore, Gelman, Sartorius membrane filters, lot number in text.

**Poured agar plate count.

A Percent of E. coli culture recovered on membrane filter on pad with M-FC medium ccmpared to filter placed on Trypticase soy agar - both at 44.5 C.

B Percent of E. coli culture recovered on membrane filter on pad with M-FC medium at 44.5 C compared to poured agar plate count on TSA at 35 C.

C Percent of E. coli culture recovered on membrane filter on TSA at 44.5 C compared to poured agar plate count on TSA at 35 C.

(M-FC pad) compared with the average counts of filters on TSA at 44.5 C. The percent recoveries ranged from 44 to 133%, with cultures 2A, 3A, and 24 providing good recoveries while cultures 1A and 3 were poor.

When the 1A culture was filtered and the membrane filters were placed on pads with M-FC medium at 44.5 C, growth occurred on Gelman lot numbers 3 and 6, but not on Millipore lot numbers 3 and 13, and Sartorius lot numbers 1 and 4. Growth was produced on all 6 lot numbers when the filters were placed on TSA medium and incubated at 44.5 C. Since this 1A culture was unusual, it will be omitted in the remainder of results for Table 1. However, further tests were conducted later on this culture (Refer to Special test - A).

The second category-B shows the percent recovery of E. coli counts on membrane filters M-FC at 44.5 C compared with poured TSA plate counts at 35 C. The percent recovery ranged from 47 to 108%, with culture numbers 1 and 9 providing best results.

The third category C presents the percent recovery of E. coli on membrane filters (TSA) at 44.5 C compared with the poured agar plate count on TSA incubated at 35 C. The percent recovery ranged from 55 to 131%, with culture numbers 1, 9 and 3Z providing best results. These results are applicable only for the lot numbers of membrane filters tested.

With respect to efficiency of each filter brand and lot number, the variations apparently were due to the culture (1A and 3), or to the filter used (S-3 with culture 2A). Since one specific culture dilution was tested at one time with the different filters, the efficiency of the latter can be compared for that particular culture. Due to lack of supply, not all filter lot numbers could be tested with all cultures and some of the results may require further tests.

Gelman lot number 3, tested with 11 cultures, had a percent recovery of 71 to 114% (Category A), 65 to 109% (Category B), and 64 to 131% (Category C). Compared with the other lot numbers, G-3 provided the best overall percent recovery for the 11 cultures tested. Lot number G-6, tested with 6 cultures, did not provide results that were as good as G-3.

Lot M-13, tested with 5 cultures had a percent recovery of 55 to 83% (Category A), 51 to 74% (Category B) and 72 to 95% (Category C). Lot M-6 had a percent recovery of 83 to 95% (Category A), 59 to 80% (Category B), and 62 to 96% (Category C). Lot numbers M-3, M-5, M-7, M-8, and M-12, each were used with only one test culture, with M-5 and M-8 producing comparable results for all categories. Lot M-12 had an unusual result with culture 24, a high (133%) recovery for Category A compared to 59% for Category B.

Lot numbers S-1 and S-3 were used with 4 cultures. Lot S-1 varied in percent recovery, having 64 to 99% (Category A), 53 to 70% (Category B), and 55 to 84% (Category C). For the same categories respectively, lot S-3 had recoveries of 44 to 87%, 47 to 82%, and 70 to 107%.

Lot numbers S-4, S-5, S-6, S-7, and S-8 each were tested with 2 cultures. Lot S-4 provided best results with culture #3A, and S-7 was good with culture #24, but not with culture #9. Lot S-5 was good with culture 2A but not quite as good with culture #9.

There were some filter lot numbers that gave nearly identical results with the same culture, such as S-5 and G-3 with culture #9, S-6 and G-6 with culture #16, and M-6 and S-3 with culture #1.

In the test with culture #1 where no filter was used, the recovery on poured agar plate counts for Categories A and B was lower than that obtained with the filters. Further tests (Special test — A) indicated a much lower count for poured agar plates with this culture.

The results in Table I are an indication of the erratic behavior of membrane filters with respect to recovery of E. coli test strains. Filtering the culture dilution and placing the membrane filter on TSA medium at 44.5 C sometimes gave a lower percent recovery (57 to 69%, G-3 and S-4, culture 3A, Category C) than when the filter was placed on M-FC at 44.5 C (102 to 104%, Category A). However, the percent recovery (Category B) of culture 3A was similar when filters were placed on M-FC (pad) at 44.5 C (60 to 70%) or on TSA at 44.5 C (57 to 69%) and compared with poured TSA plate count at 35 C.

The opposite effect was obtained when culture #9 was used with filter lot number S-5, and filter G-3 was used with cultures #9 and 4.

Comparison of E. coli Recovery on Pour Plates at 35, 43, and 44.5 C

The comparison of the percent recovery of nine **E. coli** cultures (average count of 5 replicates), obtained by the pour plate method on TSA and M-FC media incubated at 35 (air), 43 (Blue-M), and 44.5 C (GCA) is listed in Table 2. Results obtained with culture 1A are not included as it did not grow on M-FC or TSA at 44.5 C, and was markedly inhibited on M-FC at 43 and 35 C (30 colonies), but had good growth on TSA at 43 and 35 C (126 colonies).

The percent recovery on M-FC agar as compared with TSA was best for culture numbers 9, 16, and 24 at 35 to 44.5 C. The lowest percent recovery occurred when the counts of the other 6 cultures (1, 2, 2A, 3A, 3Z, and 4) on M-FC were

TABLE 2 PERCENT RECOVERY OF E. **COLI** CULTURES ON TSA AND M-FC MEDIA (POURED PLATE COUNTS[a]) INCUBATED AT 35, 43, AND 44.5 C.

% Recovery of **E. coli** on media at temperature listed.

Media Temp	E.C. No.	TSA 44.5	TSA 43	TSA 35	Media Temp	E.C. No.	TSA 44.5	TSA 43	TSA 35
TSA 44.5	1	100	100[b]	111	M-FC 44.5	1	76[c]	76	84
	2	100	97	92		2	75	72	69
	2A	100	93	100		2A	76	70	76
	3Z	100	86	86		3Z	70	61	61
	3A	100	111	113		3A	62	69	70
	4	100	101	89		4	51	51	45
	9	100	93	88		9	98	91	86
	16	100	85	92		16	104	89	96
	24	100	85	77		24	112	95	86
TSA 43	1	100	100	111	M-FC 43	1	69	69	77
	2	103	100	95		2	65	63	60
	2A	108	100	108		2A	74	68	74
	3Z	116	100	100		3Z	66	57	57
	3A	90	100	102		3A	49	54	55
	4	99	100	88		4	49	50	44
	9	107	100	94		9	93	86	82
	16	117	100	108		16	107	91	98
	24	118	100	91		24	100	85	77
TSA 35	1	90	90	100	M-FC 35	1	63	63	70
	2	109	105	100		2	59	57	55
	2A	100	93	100		2A	90	83	90
	3Z	116	100	100		3Z	52	45	45
	3A	88	98	100		3A	51	57	58
	4	113	114	100		4	53	54	47
	9	114	106	100		9	96	89	84
	16	109	93	100		16	107	91	98
	24	129	110	100		24	112	95	86

[a] Average of 5 replicates for each medium at each temperature.

[b] Average count on TSA at 44.5 C ÷ average count on TSA at 43 C.

[c] Average count on M-FC at 44.5 C ÷ average count on TSA at 44.5 C.

compared with TSA at all 3 temperatures of incubation. The percent recovery, when counts on TSA were compared with TSA at the 3 temperatures, dropped below 85% in only one instance (77%, culture #24). Although not listed in Table 2, the counts obtained for each culture on M-FC agar were cross-compared for the 3 incubation temperatures, and correlation in percent recovery was obtained as follows:

#1 – 83 to 121%
#2 – 79 to 126%
#2A – 82 to 122%
#3A – 79 to 127%
#3Z – 74 to 135%
#4 – 93 to 108%
#9 – 95 to 105%
#16 – 98 to 102%
#24 – 89 to 112%

From the results of the poured plate counts, it appears that **E. coli** cultures #9, #16 and #24 would be a good choice for a standardized test.

Comparison of E. coli Recovery on TSA, M-FC, and VRB at 35, 43, and 44.5 C

The percent recovery of 9 **E. coli** cultures as poured plate counts on TSA, M-FC, and VRB media incubated at 35 (air), 43 (Blue-M), and 44.5 C (GCA) is listed in Table 3. Cultures 1A and 4A (**Klebsiella**) which did not grow at 44.5 C on TSA and M-FC in previous tests, produced similar results on VRB media and will be excluded. As noted in prior tests (Table 2), the percent recovery of all nine cultures on TSA at the three temperatures showed close correlation (83 to 121%).

The percent recovery of M-FC compared with that of TSA, was lower at all three temperatures. Culture numbers 2, 2A, 3A, and 9 gave best results (61 to 98%), culture numbers 1 and 4 next best (54 to 80%), while cultures # 24 (21 to 40%), #3 and #16X (4 to 10%) were very low. The results obtained with VRB agar were better than with M-FC for all nine cultures. Recovery of cultures #2 and #3A on VRB were nearly identical with TSA at all three temperatures. Culture numbers 1, 2A, and 9 also had a good recovery (72 - 107%), with culture numbers 4 and 24 somewhat lower (24 to 73%) and numbers 3 and 16X the poorest (0 - 29%).

Comparision of E. coli Recovery on TSA, M-FC, and VRB at 35 and 44.5 C

Twelve **E. coli** cultures were tested using poured plate counts on TSA, M-FC and VRB media incubated at 44.5 (GCA) and 35 C (air) (Table 4). The percent recovery, based on the counts obtained on TSA at 44.5 C, was good (± 10%) for 10 of the 12 cultures. Recovery of cultures 3X and 24B were low (73 and 77%) on TSA at 35 C. When the counts on M-FC were compared with those on TSA, the percent recovery varied from 31 to 88%, with cultures #16X and #2Z lowest at 31 to 47%. Seven cultures (numbers 2B, 2Z, 16X, 16Z, 24, 24B, 24X) had a similar (± 5%) recovery on M-FC at 35 and 44.5 C when compared with TSA at 44.5 C, and at 35 C. Culture 3A was better on M-FC at 44.5 C than at 35 C (82% compared with 64%). Culture #24 had 74% recovery on M-FC at 44.5 and 35 C vs. TSA at 35 C, compared to 69% on TSA at 44.5 C.

The percent recovery, varying from 50 to 105%, on VRB was higher than on M-FC at both temperatures. Culture #16X was again low (32 to 40%) in recovery. Six cultures, numbers 2B, 3A, 3X, 3Z, 16Z, and 24 had recoveries between 71 to 105%.

Variations noted with M-FC media were apparent with VRB. Culture numbers 2B and 24 had higher percent recovery (85 to 96%) when VRB at 44.5 C was compared with TSA at 44.5 and 35 C, than when VRB at 35 C (71 - 80%) was compared in similar manner. The reverse (VRB at 35 C vs. TSA at 44.5 and 35 C) occurred with cultures 2X, 3A, 3Z, and 16Z where recovery was 82 to 105% on VRB at 35C as compared to 69 - 86% on 44.5C.

Culture numbers 2Z, 3X, 16A and 24B varied in another manner. On VRB incubated at 44.5 and 35 C and compared with TSA at 44.5 C the recovery was 75 - 100%, while the TSA at 35 C, the recovery was 63 - 77%.

Comparision of Fecal Coliform Recovery on M-FC and VRB Media at 43 and 44.5 C

Variations in the average colony counts (5 replicates) and percent recovery of fecal coliform bacteria were apparent for different brands and lot numbers of membrane filters in two different tests (Table 5). Sartorius lot 8 and Gelman lot 7 provided the best results when 20 ml of filtered creek water were incubated on 2 lots of M-FC and VRB media at 44.5 C (GCA). Millipore lots 6 and 11 gave the poorest results. While the colony counts and percent recovery of the other six lots were comparable in most cases after incubation at 44.5 C, they were one-fourth to one-half that obtained on VRB agar at 43 C (Blue-M).

TABLE 3 PERCENT RECOVERY OF E. COLI CULTURES ON TSA, M-FC, AND VRB MEDIA (POURED PLATE COUNTS)[a] INCUBATED AT 35, 43, AND 44.5 C.

		% Recovery of E. coli on media at temperature listed												
Media Temp.	E.C. No.	TSA 44.5	TSA 43	TSA 35	Media Temp.	E.C. No.	TSA 44.5	TSA 43	TSA 35	Media Temp.	E.C. No.	TSA 44.5	TSA 43	TSA 35
	1	100	83[b]	88		1	78	65	69		1	107	89	94
	2	100	91	86		2	98	89	85		2	100	91	86
	2A	100	92	92		2A	89	83	83		2A	98	90	90
TSA 44.5	3	100	96	113	M-FC 44.5	3	6	6	8	VRB 44.5	3	0	0	0
	3A	100	98	100		3A	95	93	95		3A	102	100	102
	4	100	89	92		4	65	57	60		4	60	54	56
	9	100	87	92		9	70	61	64		9	91	79	84
	16X	100	105	102		16X	8	8	8		16X	26	28	27
	24	100	109	90		24	24	26	21		24	56	60	50
	1	120	100	106		1	80	67	71		1	87	72	76
	2	110	100	95		2	84	77	73		2	108	98	93
	2A	108	100	100	2A M-FC 43	2A	86	79	79		2A	85	78	78
TSA 43	3	104	100	118		3	4	4	5	VRB 43	3	18	17	20
	3A	102	100	102		3A	89	88	89		3A	102	100	102
	4	113	100	104		4	63	56	58		4	73	65	67
	9	115	100	106		9	97	85	89		9	96	84	88
	16X	95	100	97		16X	7	7	7		16X	27	29	28
	24	92	100	83		24	29	31	26		24	49	53	44
	1	113	94	100		1	64	54	57		1	96	80	84
	2	116	105	100		2	86	79	75		2	96	88	83
	2A	108	100	100		2A	76	71	71		2A	102	95	95
TSA 35	3	89	85	100	M-FC 35	3	9	9	10	VRB 35	3	20	19	23
	3A	100	98	100		3A	84	82	84		3A	104	102	104
	4	108	96	100		4	63	56	58		4	50	44	46
	9	108	95	100		9	79	69	73		9	99	86	91
	16X	98	103	100		16X	7	7	7		16X	25	26	51
	24	111	121	100		24	37	40	33		24	57	62	51

[a] average of 5 replicates for each medium.

[b] average count on TSA at 44.5 ÷ average count on TSA at 43 C x 100.

TABLE 4 PERCENT RECOVERY OF **E. COLI** CULTURES ON TSA, M-FC, AND VRB MEDIA (POURED PLATE COUNTS)[a] INCUBATED AT 35 AND 44.5 C.

% Recovery of **E. coli** on media at temperature listed

Media Temp.	E.C. No.	TSA 44.5	TSA 35	Media Temp.	TSA 44.5	TSA 35	Media Temp.	TSA 44.5	TSA 35
	2B	100	108[b]		62	68		88	96
	2X	100	100		47	53		69	76
	2Z	100	90		39	31		86	69
	3A	100	100		82	82		80	80
TSA	3X	100	77	M-FC	79	60	VRB	100	77
44.5	3Z	100	97	44.5	60	58	44.5	85	83
	16A	100	94		61	58		75	70
	16X	100	110		36	40		59	65
	16Z	100	90		76	68		86	78
	24	100	108		69	74		85	92
	24B	100	73		72	53		86	63
	24X	100	88		56	49		77	68
	2B	93	100		66	72		74	80
	2X	90	100		55	61		82	91
	2Z	125	100		47	38		81	64
	3A	100	100		64	64		88	88
	3X	130	100		88	67		97	74
TSA	3Z	103	100	M-FC	48	47	VRB	95	92
35	16A	106	100	35	36	34	35	75	70
	16X	91	100		32	35		50	55
	16Z	111	100		70	63		105	95
	24	93	100		69	74		71	77
	24B	133	100		76	55		93	68
	24X	114	100		59	52		83	73

[a] average of 5 replicates for each medium at each temperature.

[b] average count on TSA at 44.5 C ÷ average count on TSA at 35 C x 100.

Transparent colonies were frequently observed on the S-8 and M-6 filters placed on M-FC medium lot A (H6DBXP), but not on lot B (910666). The transparent colonies were lactose negative, dextrose acid on triple sugar iron agar (BBL) and IMViC reactions were ––+–.

The results for Test 1 in Table 5 were obtained from 20 ml of creek water which normally produced 25 or more colonies. While the low counts obtained with M-FC and VRB at 44.5 C may appear insignificant, they do indicate a decided drop from the count obtained on VRB agar at 43 C.

Comparison of Fecal Coliform Recovery with Different Membrane Filter Lots on M-FC and VRB Media

In Table 6 two different volumes (10 and 20 ml) of one sample of creek water were tested with 5 lots of membrane filters on M-FC and VRB media incubated at 44.5 C (GCA). The fecal coliform counts per 100 ml water were higher (80 - 155) on VRB agar than on M-FC medium (60 - 125). The most consistent results on M-FC medium were produced by Gelman lot 7, Sartorius lot 5, and Millipore lot 7 for the two volumes tested. However, the difference in count per 100 ml, between manufacturer and lot number, are apparent (M-8, 60 - 125 vs. G-7, 110 - 120).

The highest count on VRB agar was obtained with G-7 filters, with S-5 next highest and the other three filters about equal. The M-8 filter was the only one that recovered 100% more fecal coliform on M-FC medium than on VRB agar. The

TABLE 5 PERCENT RECOVERY OF FECAL COLIFORM FROM 20 ML. CREEK WATER USING MEMBRANE FILTERS ON TWO LOTS OF M-FC MEDIA AT 44.5 C AND ON VRB AT 43[a] AND 44.5 C.

Media, incubation, lot no., count [b]

Test No.	Filter, Lot No.	M-FC 44.5 Lot A	M-FC 44.5 Lot B	VRB Agar 43	VRB Agar 44.5
1	M-6	1[b]	3	20	5
	G-7	8	9	32	8
	S-7	4	5	23	7
	S-8	6	6	25	5
2	M-11	15	24	50	23
	M-12	27	32	58	30
	G-7	30	30	56	28
	S-8	19	20	44	23

[a] 43 – actual temperature 41.8 to 43.6 C in plate.

[b] Average colony count, 5 replicates.

percent recovery of the other four lots varied from 60 to 94%.

Comparison of Fecal Coliform Recovery with Different Membrane Filter Lots at 43 and 44.5C

Results of six daily tests using 20 ml volumes of creek water with different lots of filters on M-FC and VRB media incubated at 43 and 44.5 C are summarized in Table 7. For the percent recovery of fecal coliform, the average count (5 replicates) M-FC at 44.5 C, (GCA) was compared with the averages obtained on M-FC at 43 C (Blue-M) and VRB at 43 and 44.5 C for each individual filter lot number.

Overall, the lowest percent recoveries were obtained when M-FC at 44.5 C was compared with VRB at 43 C. At times the percent recovery on M-FC at 43 C was identical with that obtained with VRB at 43 C (M-8, G-6, S-8). In the majority of tests, the percent recovery was as good or better on VRB as on M-FC at 44.5 C.

Gelman lot 7 was most consistent, and the best tested in four out of five tests.

Comparison of Fecal Coliform Recovery on Rinsed and Unrinsed Membrane Filters

In an effort to determine whether residues on the membrane filters were affecting fecal coliform counts, four different lots were rinsed in sterile phosphate buffer, pH 7.2, just prior to use. A separate beaker, containing 100 ml of buffer, was used for rinsing no more than five filters. The only difficulty encountered was the reduction in flow when the M-13 filters were transferred from the buffer rinse to the filtration unit.

As indicated in Table 8, filter rinsing improved the percent recovery of fecal coliform on M-FC media with all except the M-6 filters. The

TABLE 6 FECAL COLIFORM COUNTS[a] PER 100 ML. FROM 10 AND 20 ML. CREEK WATER, FILTERED AND INCUBATED ON M-FC AND VRB MEDIA AT 44.5 C.

		M-FC		VRB	
Filter, Lot		20 ml. 44.5	10 ml. 44.5	20 ml. 44.5	10 ml. 44.5
M-7	count/100 ml.	75	70	90	120
M-8	count/100 ml.	125	60	100	110
S-5	count/100 ml.	75	80	110	130
S-6	count/100 ml.	75	60	125	80
G-7	count/100 ml.	120	110	155	150

[a] Average of 5 replicates.

TABLE 7 PERCENT RECOVERY OF FECAL COLIFORM FROM 20 ML. CREEK WATER USING MEMBRANE FILTERS ON M-FC AND VRB MEDIA INCUBATED AT 43[a] AND 44.5 C.

		% Recovery		
Test No.	Filter Lot No.	M-FC 43	VRB 44.5	VRB 43
1	M-8	20	33	20
	G-6	50	100	50
	S-6	25	50	20
	S-7	150	150	100
	S-8	67	100	67
2	M-1	100	150	60
	M-3	29	83	45
	G-7	42	160	40
	S-2	58	117	47
3	M-5	77	93	82
	M-6	72	98	76
	G-7	90	96	84
	S-3	82	97	78
4	M-6	ND[c]	20	5
	S-8	ND	120	24
	G-7	ND	100	25
	S-7	ND	57	17
5	M-11	63	65	30
	M-12	84	90	47
	G-7	100	107	54
	S-8	95	83	43
6	M-7	78	58	41
	M-8	89	64	49
	G-7	98	92	68
	S-5	104	85	71

[a] 43 – Actual temperature 41.8 to 43.6 C in plate.

[b] Average count 5 replicates (M-FC at 44.5 C) ÷ average count 5 replicates. (media and temp. listed) x 100. For example, in Test 1, M-8, M-FC at 43 C, the 20% means that M-FC at 44.5 C recovered 20% of M-FC at 43 C.

[c] Not done.

non-rinsed filters recovered 73 - 81% of the buffer rinsed filters.

On VRB agar, the non-rinsed filters of M-6 and M-13 recovered 80% of the counts obtained on the rinsed filter, while with the G-7 and S-6 filters the percent recovery was 104% and 110% respectively. The M-13 filters gave the only consistent results (75 - 80%) on M-FC and VRB media.

Special Test - A

E. coli Grown on Surface or Embedded in Medium

Culture #1A, originally isolated as a deep blue colony on M-FC medium incubated at 43 C, produced few colonies on TSA medium and none on VRB and M-FC agars at 44.5 (GCA) as poured plate counts. This #1A culture grew best on TSA plates with marked inhibition noticeable on M-FC and VRB when incubated at 43 and 35 C.

In a repeat test, culture #1A was plated using M-FC and TSA agars (5 replicates each). One ml of diluted culture was used for 15 x 100 mm plates and 0.1 ml of the same dilution and a more concentrated suspension was used with two sets of 12 x 50 mm plates. No growth occurred on any of the poured plates after overnight incubation at 44.5 C in the (GCA) water bath.

In contrast, the #1A culture on membrane filters and pads with M-FC medium that were incubated at 44.5 C had growth on Gelman lot numbers 3 and 6, but not on Millipore lot numbers 3 and 13 and Sartorius lot numbers 1 and 4 (Table 1). Growth was produced on all six lot

TABLE 8 FECAL COLIFORM RECOVERY FROM 20 ML. CREEK WATER USING BUFFER RINSED OR NOT RINSED MEMBRANE FILTERS ON M-FC AND VRB MEDIA INCUBATED AT 44.5 C.

		M-FC – Pad		VRB – Agar	
Filter, Lot No.		No Rinse	Rinse	No Rinse	Rinse
M-6	Mean count [a]	14	12	16	20
	%[b]	117		80	
M-13	Mean count	12	16	20	25
	%	75		80	
G-7	Mean count	26	32	27	26
	%	81		104	
S-6	Mean count	16	22	23	21
	%	73		110	

[a] Average of 5 replicates

[b] % = Mean counts, no rinse ÷ rinse, x 100

numbers when the filters were placed on TSA medium and incubated at 44.5 C.

The average colony count of 0.1 ml of concentrated suspension of **E. coli** #2, filtered, and incubated on TSA agar at 35 C was 115, while that on an M-FC (pad) was 101. The average colony count of the 1 ml filtered and placed on a pad containing M-FC broth and incubated at 35 C was 179. Due to excess moisture and the volume (0.1 ml) streaked on the 12 x 50 mm plates, the bacterial growth ran together and counts were too erratic to be used.

Culture #2 was tested by filtering (M-13) 0.1 ml and 1 ml of two different concentrations and placing the filters on 12 x 50 and 15 x 100 mm plates of M-FC and TSA. Then the same volumes (0.1 and 1.0 ml) were respectively spread on M-FC and TSA agar plates (12 x 50 and 15 x 100 mm). Five replicates of each category were incubated in a 44.5 C (GCA) waterbath.

The agar plates that were streaked or received the filters (1 ml) had average colony counts (44.5 C) as follows (**E. coli** #2):

E. coli #2	12 x 50	15 x 100	Media
1 ml (filter)	119	134	M-FC
1 ml (filter)	169	204	TSA
1 ml (streaked)	omitted	73	M-FC
1 ml (streaked)	omitted	71	TSA

The average counts (71 - 73) of the surface streaked (1 ml) plates therefore were much lower than when the filters were placed on agar or pads.

E. coli #2, in two concentrations, was then tested by the pour plate method using M-FC and TSA agars in 12 x 50 (0.1 ml) and 15 x 100 (1 ml) plates incubated at 44.5 C (GCA bath). The colony count of #2 culture in the M-FC agar with both concentrations and plate sizes was about one-half that which occurred with the TSA agar. Repeating the experiment with the #2 culture gave the same results with poured plate counts.

One ml of **E. coli** culture #1 was then tested, as mentioned above for #2, using filters on agar (12 x 50 mm plates), and agar poured plates (15 x 100 mm). The results (average colony counts) were as follows:

Filter	M-FC	TSA	Temperature
M-6	20	24	44.5 C (GCA)
G-5	27	32	44.5 C (GCA)
S-8	20	30	44.5 C (GCA)
S-3	20	23	44.5 C (GCA)
Poured	14	27	44.5 C (GCA)
Poured	10	25	35 C incub.

When 0.1 ml of concentrated #1 suspension was poured in 12 x 50 plates, the average count on M-FC was 12 and on TSA, 24. Using 15 x 100 plates, the poured plate counts were 5 on M-FC and 22 on TSA. Incubation was 44.5 C (GCA) in both tests.

It should be noted that where two concentrations of a culture were used, the counts are comparable only with one or the other concentration.

There was, therefore, a difference between placing the culture on the surface of the agar medium via a filter or by spreading the liquid and incorporating the culture in the medium as in the poured plate method with **E. coli** #1, #1A, and #2.

Special Test - B

Tests were conducted to determine: 1) whether the bacteria are trapped on the filter surface but not all will grow, 2) some are trapped deep in the membrane and would grow through the other side (bottom of filter), and 3) some of the bacteria actually escape through the membrane into the filtrate. It was assumed that all bacteria trapped on the surface of a membrane filter would be transferred by contact to the surface of an agar plate . The contact time used was one, two, and four hours, or the filter was left in place.

Two cultures, #2 and 3A, were diluted and 1 ml (5 replicates for each) filtered through Gelman, Millipore, and Sartorius membrane filters. For comparison, 1 ml was also plated with TSA for pour plate counts and incubated at 44.5C and 35C.

One group of filters was placed upside down and a second group right side up on corresponding pads saturated with M-FC medium. In a similar manner the same brand and lot numbers of filters were placed on M-FC agar (Test A) or VRB agar plates (Test B) and incubated at 35 C and 44.5 C. One hour later, some of the upside down filters were removed from the plates, with others left in place. This procedure was again repeated after four hours incubation, and all plates returned to their respective incubation overnight (Table 9).

No bacterial colony growth was evident on either side of the filters placed upside down on the pads or from the filtrates. However, the colonies present on filters upside down on the agar plates could be counted through the agar. The colony counts on plates (and percent recovery) with filter removed after one hour incubation were lower than those removed four hours later. The latter colony counts were higher than where filters were left on the plates, (Test A, Table 9), with the exception of culture 3A on Millipore lot #13. However, the recovery of **E. coli** by the filters on the pads and agar plates did not equal the poured plate counts on TSA medium for culture 2 and 3A on M-FC at 35 C and 44.5 C.

In test B (Table 9), when the same filters and cultures were used, there was a marked increase in present recovery of culture #2 with filters placed on VRB agar at 35 and 44.5 C. A slight increase occurred when filters were removed after two hours. However, results of **E. coli** #3A on VRB agar were about the same as with M-FC medium.

DISCUSSION

To determine efficiency of membrane filters for the fecal coliform test one must test polluted water samples, and confirm that the colonies are fecal coliform. To detect deficiences in the test procedure, **E. coli** strains are used, but these vary in different reports (1, 5, 6, 8). However, all test methods and equipment must be accurate and rigidly adhered to. The water baths and incubators used must maintain a temperature of 44.5 C **inside the plate**, but actual reports are scarce. The GCA - Precision Scientific Co. water bath was the only

TABLE 9 COLONY COUNTS AND PERCENT RECOVERY ON MEMBRANE FILTERS PLACED ON PADS AND AGAR MEDIA AND REMOVED OR LEFT IN PLACE.

			M-FC Medium or agar plate and incubation[b]									
Test A Culture	Filter[a] Lot No.		Pad		1 hr.		4 hr.		Left on		TSA[c]	
			44	35	44	35	44	35	44	35	44	35
2	G-6	count	43	55	21	31	47	57	18	23	72	70
		%[d]	60	79	29	44	65	81	25	33	100	100
2	S-4		35	32	17	33	36	35	30	22		
		%	49	46	24	47	50	50	42	31		
2	M-13		49	51	27	40	51	47	31	32		
		%	68	73	38	57	71	67	43	46		
3A	G-6		74	77	23	24	75	86	50	72	104	112
		%	71	74	22	21	72	77	48	64	100	100
3A	S-4		57	67	54	44	43	63	54	68		
		%	55	60	52	39	41	56	52	61		
3A	M-13		61	73	65	38	63	76	51	65		
		%	59	65	63	34	61	68	49	58		

					Filter Removed							
Test B Culture	Filter[a] Lot No.		VRB		2 hr		4 hr		Left on		TSA[c]	
			44	35	44	35	44	35	44	35	44	35
2	G-6	count	40	48	56	58	ND		ND		50	52
		%	80	92	112	112					100	100
2	S-4		44	54	58	55	ND		ND			
		%	88	104	116	106						
2	M-13		47	49	46	60	ND		ND			
		%	94	94	92	115						
3A	G-6		24	23	18	18	ND		ND		31	28
		%	77	82	58	64					100	100
3A	S-4		18	20	16	18	ND		ND			
		%	58	71	52	64						
3A	M-13		18	14	15	15	ND		ND			
		%	58	50	48	54						

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[a] Gelman, Sartorius, Millipore (average of 5 replicates).

[b] Filters removed after 1 hr, 4 hrs, or left on plate.

[c] Poured Trypticase soy agar plate count.

[d] Percent recovery, count at 44 ÷ TSA count at 44.5 C.
35 ÷ TSA count at 35 C.

ND = not done.

one used in our tests that kept a steady 44.5 C temperature inside the plate and the surrounding water.

Three incubation temperatures were utilized to determine differences in percent recovery. The number of bacteria that grew on TSA medium at 35 C should be an indication of the number of E. coli present in the dilution being tested. From this base, comparisons with counts obtained at 43 and 44.5 C were made, not only with TSA medium but also with M-FC and VRB. The 43 C incubation temperature (41.8 to 43.6 C inside plate) was used, as we had noted these temperatures in improperly working water baths and air incubators set at 44.5 C.

In prior reports, the exact number of bacteria cells being tested was based on poured plate counts (8), or on counts obtained on membrane filters with pads, using a non-inhibitory medium (5). That either or both of these methods could effect the results obtained with E. coli cultures was apparent in our study.

The results we obtained indicated that eleven of the **E. coli** test strains varied not only in their ability to grow at 35, 43, and 44.5 C, but also on or in the culture media used. Culture 1A would grow on TSA agar, but not on M-FC agar at 44.5 C. Theoretically, a temperature tolerant culture should grow as well at 35 C as at 44.5 C on a non-inhibitory (TSA) medium, but this did not always occur (Table 1, cultures 2 and 9). At times, the percent recovery of membrane filters on M-FC vs. TSA at 44.5 C was better than that obtained on TSA at 44.5 (C) and 35 C (B), (Table 1, cultures 2A and 3A). This is similar to the results obtained by Presswood and Brown (8) with three **E. coli** cultures. However, while they used M-FC medium for both the membrane filter and poured plate counts, there is agreement that the membrane filter was responsible for low percent recovery more frequently when M-FC medium was used.

When the **E. coli** strains were tested by the pour plate method, the percent recovery of nine cultures on TSA medium was very close for the three temperatures of incubation (Table 2). In contrast, only three cultures (9, 16, 24) grew as well on M-FC as on TSA at the three temperatures of incubation. The addition of VRB medium for comparison with TSA and M-FC media by the pour plate method indicated that VRB had a better percent recovery than M-FC (Tables 3, 4). Culture #9 again produced good results, but in contrast culture #24 showed inhibition on M-FC and VRB agar. This would indicate variations of temperature tolerance in some of the test cultures.

A comparison of VRB agar with M-FC medium in respect to recovery of fecal coliforms from polluted water indicated that VRB was better than M-FC when the incubation temperature was 44.5 °C (Tables 5, 6, 7), and quite superior at 43 C (Table 5).

The possibility that the membrane filter-culture media interaction was responsible for low recovery was obvious when the membrane filter counts were compared with poured plate counts. An attempt to transfer bacteria on the membrane filter to M-FC medium did not improve percent recovery as well as on VRB medium (Table 9). Placing three lot numbers of filters containing **E. coli** #2 on VRB agar at 35 and 44.5 C produced much better results than on M-FC with pads (Table 9, Test B). An improvement in percent recovery was also noted when the same culture and membrane filters were placed face down on the VRB agar, and then removed two hours later. However, when **E. coli** #3A was tested in the same manner, there was little difference in percent recovery on VRB and M-FC media. The results obtained on M-FC with **E. coli** #2 and #3A (Table 9, Test A) are similar to those obtained in Table 1 (Category B). Therefore, we must assume that VRB was better than M-FC, at least for culture #2 in this test. Whether placing the membrane filter face down on the VRB agar and stimulating better growth by direct contact can be compared with the two layer technique (7, 9) requires further study.

Temperature alone did not affect the growth of the nineteen **E. coli** strains tested in an equal manner since all grew well on TSA medium. The **E. coli** strains were affected more by their ability to form less colonies on M-FC agar than on TSA and VRB media. Differences in strains or isolates, with some showing no growth inhibition while others were markedly inhibited, were apparent. It therefore appears that the M-FC medium did support good growth of some, but not all, of the **E. coli** strains. This applied to pour plates as well as membrane filter tests. Since one lot medium was used for most all tests, different lot numbers were not responsible.

A comparison of different brands and lot numbers of membrane filters indicated variations in percent recovery of **E. coli** cultures were due to the filter, the culture, or the test method. Culture #1 as poured plate count recovered 52 to 58% of the E. coli on M-FC when compared to TSA at 44.5 and 35 C (Table 1), and similar results were obtained with poured plate counts (Special Test A). In each of these two tests, the membrane filter counts were higher (67 - 109%) and membrane filters M-6, S-8, and S-3 produced identical results in respect to percent recovery (67 - 87%) at 44.5 C. Cultures #1A and 2 also produced lower counts when tested by the poured plate method and compared with membrane filter counts. The M-FC agar was more inhibitory (lowered counts) than the TSA for these cultures via the membrane filter or poured plate method. With culture #2, this inhibitory activity of M-FC was less marked when streaked on the solidified M-FC and TSA plates, even though the surface streaked count was much lower than the poured plate count (Special Test A).

Therefore, in evaluating the efficiency of the membrane filters in respect to percent recovery of E. coli bacteria, the ability of the test strain to grow in or on the medium should be considered. In Table 1, when the membrane filters were placed on M-FC medium (pads) or on TSA agar, Category A probably provides a fair comparison of the different lot numbers. That the percent recovery might be due to the method, i.e. Category C, where the membrane filter was placed on TSA (44.5 C) and compared with poured plate count on TSA (35 C) is evident with cultures #1 and #9 vs. #2 and #3A. All four cultures (in fact all cultures tested), were almost equal in percent recovery when compared at 44.5 C and 35 C on a poured plate basis (Tables 2, 3, 4). In this respect, there was little difference reported in bacteria counts obtained when membrane filters were tested with plate count broth and total coliform broth at 35 C, but a decided drop occurred in the counts when plate count broth and M-FC broth were used at 44.5 C (5).

Why the membrane filters are so erratic is still not clear. Rinsing the filters prior to use helped somewhat in limited tests (Table 8). The bacteria trapped on the membrane filter surface required more than one hour contact time with a solid culture medium to grow. It is possible the bacteria on the filters may be in clusters, or somehow injured and do not grow as readily as in poured agar plates. However, we did find two strains of E. coli that grew better on the surface of the medium than in the poured agar. If this was due to melted agar being too hot, all samples done at that time would have been affected, but this did not occur.

While it appears that VRB medium might be as effective or better than M-FC medium, this should be evaluated by others. Klein and Fung (6) reported that the VRB poured plate method was as good as the MPN and membrane filter method for the fecal coliform tests. However, an air incubator at 44.5 C and only two replicates per sample were used in their tests.

SUMMARY

The conclusions to be drawn from this study emphasize that a test method should be standardized with respect to the E. coli strain, the culture medium used to determine the actual number of bacteria in the dilution being tested, the best method to be utilized (poured plate, pad and broth, or solidified agar plate) and control of temperature of incubation inside the medium. Studies to further standardize the culture medium to be used at 44.5 C have been initiated (9) and hopefully this will provide more uniform blue colony types.

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RECOVERY CHARACTERISTICS OF BACTERIA INJURED IN THE NATURAL AQUATIC ENVIRONMENT

by

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ABSTRACT

The recovery and enumeration of indicator organisms from natural waters were found to be adversely influenced by several phases of the system. These dictated the degree of sub-lethal injury observed in water-borne bacteria that rendered them incapable of growth under conditions that are routinely used for their identification. Therefore, the recovery of indicator organisms following water-induced injury was examined using various isolation procedures. A high degree of variation was found and the membrane filtration technique was the least efficient. The injury inflicted upon **E. coli** and **S. faecalis** by the aqueous environment could be reversed by a short enrichment treatment. This procedure was especially applicable to membrane filtration procedures.

INTRODUCTION

In evaluating the problem of detecting particular microorganisms from various sources, proper consideration must be given to the influence of environmental factors upon detection methods. Data are available indicating that after exposure to freezing, heating, or freeze-drying, some microorganisms are either physiologically debilitated or injured to such an extent that significant problems arise upon attempts at detection and enumeration. Such stress-injured microorganisms become more sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies.

Most sanitary indicator organisms and enteric water-borne pathogens are bacteria whose natural habitat is the intestine of man and warm-blooded animals. Once these microorganisms are deposited into water they are in an environment that is not favorable to the maintenance of viability for most heterotrophic bacteria. Therefore, proper interpretation of sanitary water quality data relies partly on a basic understanding of survival characteristics of bacteria in water. In the majority of reported survival studies, only two sub-populations of the total have been considered: those cells which can withstand the aquatic environment as reflected by their detection and enumeration when using standard laboratory procedures and conversely, those cells which cannot persist in the unfavorable environment, resulting in death and non-detection. There is a paucity of available literature concerning the possibility that a substantial fraction of the total population of cells in water may be injured to the extent that they fail to grown on selective media.

This report presents research directed toward determining whether aquatic environments provoke stress upon indicator bacteria such that these cells become physiologically debilitated and cannot be detected by direct selective procedures. The report also describes methods to recover these injured cells.

In the first study, membrane chambers were filled with washed suspensions of a typical EC+ strain of **Escherichia coli**, type I, and immersed at different stream sites, and sampled daily. The organisms were tested for their ability to form colonies on Trypticase soy agar supplemented with yeast extract (TSY agar), to indicate the maximum number of recoverable organisms, and on desoxycholate agar (DLA), to yield the number of bacteria that were able to form colonies in the presence of this selective medium. From these data, the percentage survival and the percentage death were obtained in addition to the fraction of the total viable population that was injured to the extent that they were unable to grow on the selective medium. This portion of the total viable population that became debilitated in water represents the cells that would not be enumerated using the current standard procedures.

The results of the first study indicated that the number of organisms in these segments of the total bacterial population varied considerably among the stream sites that had different water quality characteristics. Also, at the sites where the greatest percentages of death was observed, there was a greater proportion of the bacteria that were injured. The extent of this non-lethal injury varied from 10% to 96% of the total viable population after four days, depending on the physical and chemical characteristics of the water. In addition, it was observed that the proportion of the survivors that reflected non-lethal injury increased as the length of exposure to the aquatic environments increased. This observation was also found when comparable experiments were conducted using **Streptococcus faecalis**.

Because the isolation of various indicator bacteria is the primary objective in assessing the microbiological quality of water, the use of selective media is required to suppress the growth of other organisms that could interfere with the detection and enumeration of the desired microorganisms. Inhibitory agents in these media may exert unexpected inhibition on cells subject to stress. Thus, the combination of environmental stress with the subsequent utilization of selective media may result in the diminished recovery of injured cells. This problem is further complicated by the varying degrees of injury observed as a function of time and water characteristics. Therefore, improved enumeration methods should be developed to more efficiently recover injured bacteria.

In the second study, experiments were done to establish the relative efficiency of various selective media in recovering **E. coli** that were progressively debilitated in natural water. Samples were enumerated daily for four days by different methods using several media. These studies revealed the superior recovery efficiency of liquid media (MPN) over broth plating and membrane filtration methods: MPN with TSY = MPN with lactose broth > MPN with brilliant green lactose bile broth > plating with desoxycholate lactose agar > membrane filtration with M-Endo MF medium > membrane filtration with M-FC medium. It should be emphasized that the membrane filter procedures were less efficient in recovering injured bacteria found in natural waters than the other selective procedures by a statistically significant margin. Similar results were found when comparable experiments were done using **S. faecalis** and the appropriate media. These findings indicate that, while conventional methods might be adequate to enumerate bacteria from water exerting minimal environmental stress or cells deposited into the water shortly before it was sampled, careful consideration should be given to the development of new methods to recover debilitated bacteria from stressful water environments. This is particularly true, since injured cells were found to be a large fraction of the total population when exposed to certain aquatic environments for as little as two days. Therefore, it is important that this damaged population be recovered to more correctly evaluate the bacterial quality of many waters.

In the third study, experiments were conducted to determine if cells injured by environmental stress in natural waters had the capacity to repair themselves when exposed to a suitable environment. An EC+ strain of **E. coli** was exposed to water in membrane chambers for two days, the organisms removed, inoculated into liquid TSY medium and enumerated every hour by plating on TSY and DLA agar for six hours. This procedure was used to compare the growth kinetics of bacteria exposed to water with a control suspension of the same organism that was not subject to environmental stress. As in the previous experiments, the difference between the counts on the TSY and DLA media reflected the debilitated population. Control cultures that were not exposed to the water contain virtually no cells in this weakened physiological state and the bacteria exhibited normal growth kinetics. However, the cell suspension that had been exposed to the stresses of the aquatic environment for a period of two days contained a substantial proportion (95%) of injured cells, and the lag period was three times longer than in the control suspensions. As the injured cells were exposed to the TSY broth, the injured population progressively repaired itself, so that after three hours, they were capable of producing colonies on both TSY and DLA agar. Further experiments, where bacteria were in the water for longer times, demonstrated increases in the lag period and recovery time. The same kinds of observations and relationships were found when similar experiments were conducted using *S. faecalis*. These studies indicate that appreciable repair from environmental injury may be attained by exposing the cells to a rich, non-selective medium for a short period prior to the use of a selective medium. This procedure affords the more complete enumeration of indicator bacteria from the aquatic environment on selective media. Enrichment techniques appear to be especially applicable to membrane filtration methods that have a low efficiency of recovery for injured cells and are easily adapted to enrichment procedures.

Additional studies were conducted to determine if a two hour enrichment step, before exposure to the selective medium, would enhance the recovery of total and fecal coliform bacteria from a mixed natural population of bacteria, as they were in contact with the aquatic environment for various times. In these experiments, suspensions of raw sewage were placed in membrane chambers and then in a stream, followed by daily sampling using the membrane filter procedure. Then duplicate filters were incubated on M-Endo agar at 37 C and M-FC agar at 44.5 C, after a two hour enrichment step on TSY agar at 37 C. These experiments indicated that the enrichment procedure improved the recovery, in comparison to control samples, where the enrichment was omitted. Throughout the entire three day period that the bacteria from the sewage were in contact with the aquatic environment, the most efficient method for recovering total and fecal coliform bacteria was a two hour enrichment on TSY agar followed by transfer to the selective medium. The two hour enrichment period provides a non-toxic, nutrient rich environment for the gradual adjustment and repair that is needed by these organisms to successfully grow on the selective medium. The adoption of enrichment techniques would help overcome some of the limitations of the membrane filtration procedure, arising from the exclusive use of selective media, by providing the necessary bridge for bacterial adaptation between the environment encountered in natural waters and the selective media in the laboratory.

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A LAYERED MEMBRANE FILTER MEDIUM FOR IMPROVED RECOVERY OF STRESSED FECAL COLIFORMS

Robert E. Rose, Edwin E. Geldreich and Warren Litsky

ABSTRACT

A two layered agar method employing temperature acclimation and lactose enrichment with diffusion transfer into M-FC agar is proposed for improved recovery of stressed fecal coliforms on the membrane filter. The procedure was field tested in three laboratories using samples of raw and chlorinated wastewater, reservoir, river and marine waters. Verification of 1013 fecal coliform colonies isolated from 61 water samples averaged 92 percent using this proposed procedure. Comparisons with the Standard Methods M-FC procedure revealed the two-layered agar method, provided an overall increased sensitivity for fecal coliform detection in chlorinated secondary effluents, marine waters and any natural waters that contained pollutants with heavy metal ions.

INTRODUCTION

Recent reports of reduced recovery of fecal coliforms from chlorinated sewage effluents, when the membrane filter procedure is used (1-3), have caused much concern both to regulatory agencies and thos laboratories involved in monitoring. Until this problem is resolved, Federal requirements for the bacterial quality assessment of effluents under the National Pollution Discharge Elimination System has specified that fecal coliform densities must be determined by the multiple tube procedure. As a result of this decision, many small laboratories are unable to meet this analysis protocol because of a limited bacteriological testing capability based solely on the membrane filter concept.

Review of the attenuated fecal coliform recovery problem suggested that chlorine inactivation of some coliform cells might be reversed provided enrichment (4, 5) and temperature acclimation (5, 6) were possible without compromising specificity of the test. All enrichment procedures, previously developed for the membrane filter technique, required a manual transfer of the membrane filter cultures from one medium to another (4-9). Recognizing media manipulations is time consuming in the laboratory so a new approach incorporating a two layer enrichment-differential growth medium was explored. The method was evaluated on a variety of waters which might contain attenuated fecal coliforms.

MATERIALS & METHODS

Preparation of a two layer medium (Table 1) was accomplished by dispensing approximately 5 ml of M-FC agar into each culture dish (50 x 12mm), permitting the agar to solidify, then adding 2 ml of normal strength lactose broth in 1.5 percent agar over the M-FC agar. Since the ingredients of the two agar layers will eventually diffuse

TABLE 1. FORMULATION OF THE TWO-LAYER MEDIUM

Differential Medium (Botton Layer)	3.7 gm
M-FC Medium	1.5 gm
Agar	100 ml
Distilled Water	
Resuscitation Medium (Top Layer) *	0.3 gm
Beef Extract	0.5 gm
Peptone	0.5 gm
Lactose	1.5 gm
Agar	100 ml
Distilled Water	

*Resuscitation medium equals 1x lactose broth plus 1.5% agar.

into each other, it is suggested that the base M-FC agar be prepared in advance and the lactose agar overlay added within one hour prior to use.

After the membrane filter was placed on the two-layer medium, the plates were incubated at 35 C for 2 hours after which the temperature was increased to 44.5 C for 22-24 hours to attain the necessary selectivity. All blue colonies were counted with the aid of a binocular scope employing 10-15x magnification and a flourescent light source. Verification of fecal coliforms isolated on the test medium was performed by subculturing each blue colony into either phenol red lactose broth or lauryl tryptose broth for 24 to 48 hours at 35 C. Tubes showing gas production within this period were subcultured to E.C. broth and incubated in a water bath for 24 hours at 44.5 C ± 0.2 C.

Samples were collected from diverse waters that included estuarine waters of Massachusetts, raw sewage and chlorinated sewage effluents from the Billerica Massachusetts sewage treatment plant, polluted stretches of the Merrimack, Fort and Mill rivers, and sampling at varying depths of a raw water impoundment near Walton, Ky. The reservoir water samples were collected during a period of prolonged dry weather and following a significant stormwater runoff into the impoundment. All bacteriological examinations of these waters were performed at one of three different laboratories located near the sampling sites: Millipore Corporation Laboratory at Bedford, Mass.; University of Massachusetts Department of Environmental Sciences Research Laboratory at Amherst, Mass.; and the US EPA Water Supply Research Laboratory, Cincinnati, Ohio. Three or five replicate portions were prepared for cultivation on both the two-layer experimental medium and the M-FC agar direct method as recommended in Standard Methods (10).

RESULTS AND DISCUSSION

A total of sixty-one water samples were analyzed in the evaluation of the two-layer medium procedure. The choice of samples used in this evaluation were oriented to those waters that might have attenuated fecal coliform populations resulting from exposure to chlorination of sewage effluents, contact with the marine environment, antagonistic action of metal ions in chemically polluted fresh waters and to natural forces of self-purification induced during storage of impounded natural waters. Data presented in Tables 2 to 5 are based on average colony counts per membrane filter test rather than as counts per 100 ml so as to avoid distortion from factoring dilutions to the base 100 ml level.

The fecal coliform colony counts on the two-layer method were greater than those detected by the companion direct M-FC procedure when chlorinated sewage effluents were examined (Table 2). The average colony count ratio obtained by the experimental procedure and the standard M-FC method was 18.2. Inspection of the comparative data for raw sewage revealed only three of 18 samples had a ratio higher than the lowest ratio calculated in the chlorinated sewage effluents. These preliminary findings suggest attenuated fecal coliforms are more numerous in chlorinated

TABLE 2. COMPARISON OF THE 2-LAYER AGAR VS DIRECT M-FC PROCEDURES COLIFORM DENSITIES FROM RAW AND CHLORINATED SEWAGE

Source	M-FC Count	2-Layer Agar Count	Ratio 2-Layer Agar Direct M-FC
			1.8
Raw Sewage	57	103	1.1
	23	26	2.3
	32	72	1.3
	16	20	1.6
	16	26	3.1
	10	31	2.8
	4	11	10.0
	1	10	7.0
	2	14	2.0
	46	91	2.4
	5	12	1.5
	15	23	1.4
	24	33	1.5
	15	22	1.7
	25	42	1.9
	52	98	2.7
	12	32	5.7
	7	40	
			Avg. 2.9
			38.0
Chlorinated Sewage	6	228	26.0
	1	26	4.9
	26	127	3.8
	5	19	
			Avg. 18.2

sewage than might be expected in raw sewage and thus support the observations of Lin (1).

Attenuated fecal coliforms also appear to be present in the estuarine samples collected from a coastal site in Massachusetts (Table 3). Stevens et al. (9) were of the opinion that the initial shock at 44.5 C adversely affected reproduction of metabolically injured cells present in the marine water environment. Here again, the two layered medium recovered from 3.8 to 7 times more fecal coliform colonies than the direct M-FC procedure.

TABLE 3. COMPARISON OF THE 2-LAYER AGAR VS DIRECT M-FC PROCEDURES FOR FECAL COLIFORM DENSITIES FROM MARINE WATERS

Source	M-FC Count	2-Layer Agar Count	Ratio 2-Layer Agar Direct M-FC
			4.0
Marine Waters	3	12	3.8
	21	79	4.0
	3	12	3.8
	21	79	5.3
	3	16	7.0
	30	210	4.6
	20	92	
		Avg.	4.6

Attenuated fecal coliform occurrences in fresh waters are more varied as related to the intensity of heavy metal ions found in a particular stretch of river (Table 4). The toxic effect of heavy metal ions in river water has been reported to be a factor in coliform recovery from transported samples (11-13). Adsorption of metal ions from a water sample may also occur on the membrane filter, producing a concentrated toxic effect (11). Thus, the more frequent occurrence of attenuated coliforms observed in the Merrimack River, as contrasted to data obtained on Fort River, is assumed to be a reflection of the more numerous industrial waste discharges to the Merrimack River.

TABLE 4. COMPARISON OF THE 2-LAYER AGAR VS DIRECT M-FC PROCEDURES FOR FECAL COLIFORM DENSITIES FROM RIVER WATERS

Source	M-FC Count	2-Layer Agar Count	Ratio 2-Layer Agar Direct M-FC
Merrimack			6.3
River	4	25	2.4
	76	183	6.3
	4	25	2.4
	76	183	2.3
	23	53	2.5
	15	38	7.0
	8	56	
			1.9
Fort River	11	21	2.8
	4	11	1.4
	7	10	1.8
	9	16	1.2
	24	29	
			2.0
Mill River	6	12	2.7
	22	60	
		Avg.	3.1

Results obtained from a study of a water reservoir supply (Table 5) indicate the least amount of difference between the proposed two layered medium and the direct M-FC medium. This water contained few fecal coliforms per 100 ml during the day weather and no industrial waste discharge in the drainage basin. Fecal pollution that enters from stormwater runoff is from cows grazing on the hills surrounding the reservoir. For these reasons, those few attentuated fecal coliforms present, represent debilitated cells common to natural die-off.

Verification of 1013 typical blue colonies (Table 6) from the two-layer agar procedure confirmed our contention that the reported increased fecal coliform recoveries attributed to this proposed procedure were valid. Of 1013 colonies picked from all samples tested, 930 produced gas at the elevated temperature, for an average verification rate of 92%.

CONCLUSIONS

The results indicate that the proposed two-layer agar membrane filter procedure allows for

TABLE 5. COMPARISON OF THE 2-LAYER AGAR VS DIRECT M-FC PROCEDURES FOR FECAL COLIFORM DENSITIES FROM RESERVOIR WATER

Source	M-FC Count	2-Layer Agar Count	Ratio 2-Layer Agar Direct M-FC
Water Supply			0.9
Reservoir	11	10	1.8
(Dry Period)	17	30	1.0
	44	44	1.3
	10	13	1.5
	21	31	0.9
	94	89	1.7
	6	10	1.3
	8	10	1.2
	36	43	
			Avg. 1.3
Water Supply			1.1
Reservoir	91	96	1.4
(Storm Water	32	44	1.1
Runoff)	86	95	1.1
	100	106	1.2
	140	170	1.3
	80	100	1.3
	95	125	1.1
	53	55	1.2
	130	160	
			Avg. 1.2

TABLE 6. VERIFICATION OF BLUE COLONIES FROM TWO-LAYER CULTURES

Source	Number of Colonies Subcultured	Colonies as Verified Fecal Coliforms	Percent Verification
Raw Sewage	538	477	88.7
Chlorinated Effluent	70	69	98.6
River Water	145	132	91.0
Marine Water	80	79	98.8
Reservoir	180	173	96.1

repair and subsequent reproduction of those fecal coliform which have been debilitated by exposure to chlorine, industrial waste or marine waters. The decision to use the slightly more involved two-layered medium procedure in preference to the direct M-FC method should be based on a demonstration of increased verified recovery of fecal coliforms from samples routinely examined. With a major interest in the fecal coliform test being related to the bacterial quality assessment of effluents, the proposed technique should be considered as an alternative Standard Methods fecal coliform membrane filter test, specifically intended for those waters known to have significant levels of attenuated fecal coliforms.

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QUESTIONS AND ANSWER SESSION

Lin. Are there any shortcomings when you combine two media in one?

Geldreich: Yes. By putting a top layer as an enrichment medium in that position, within two hours if diffuses into the M-FC. They diffuse together and become the same medium. At the beginning, the membrane is separated from the more specific medium, the M-FC medium, by this layer of an enriched material. We used the procedure many years ago when we were trying to work out an overlay concept for pour plates. We were at one time hoping that we could come up with a coliform pour plate approach which would be cheap and wouldn't use membranes and other materials. That is the concept. Now you cannot lay the top layer on more than an hour in advance, because it will diffuse into the other medium and you will have the fused media and you won't have the advantage of an automatic transfer concept.

Dufour: I would like to know what percentage of debilitated organisms you are recovering. In other words, what were you using as the best estimate, or why didn't you use the MPN for instance, as the best estimate?

Geldreich: We are using MPN. The material is so preliminary in nature that I didn't have time to add the MPN results. That is being done for the ASM meeting.

Dufour: What bothers me is the fact that you may be getting seven times more recovery but if your regular M-FC count is down by two logs it really doesn't make much difference.

Geldreich: Well, we will be able to give you more information related to a base of an MPN in May in the ASM.

McFeters: Is your verification data based strictly upon bacteria that were isolated that appeared as fecal coliforms from the medium?

Geldreich: I would like to have done what some of the rest of you have done but I didn't have time. That is to take some of these organisms out of the environment, which you know you are having trouble with, or pure cultures which you know don't work too well, put them through the system and then look at it. We didn't have this time as of now.

McFeters: I just wondered about the background of non-fecal coliforms.

Geldreich: We had very little background on all samples except those which were in the reservoir. The reservoir samples occasionally gave us an increased background of other bacteria which were white colonies. That is one of the difficulties when you begin playing around with changing temperature and media enrichment. You are going to increase the risk of getting some bacteria that you don't want as background.

Ginsberg: The instructions for performing the M-FC test state, rather adequately, that the plates must be incubated within 10 to 20 minutes after filtration. Now, you are suggesting that you incubate at the lower temperature for two hours. How do you explain this contradiction?

Geldreich: I explain this for the very reason that we are trying to get an enrichment started at 35 C, not at room temperature. If you lower the temperature further to room temperature, we are going to have more and more problems with background organisms. I would like to avoid completely, any use of a lower temperature, but we need a temperature acclimation, and I think what you are going to see may be in the next paper. I've talked to Jack Delaney and Grasso and they have come to the conclusion, in their preliminary work, that one of the problem is a shock of these organisms when coming out of a cold environment and suddenly put at a jarring 44.5 C temperature. Maybe they need a period of acclimation.

Brezenski: I am a little disturbed. If I read you correctly, you said this is a proposed method to substitute the routine membrane filter procedure for chlorinated effluents. In Figure 2, you showed four chlorinated effluent samples and I think that three out of the four were below the valid statistical range. I'm happy to see something like this but I am afraid that I don't see enough data to make a proposal at this time, based on this amount of data. Are the sewage effluents chlorinated?

Geldreich: As I told you when I was reading the paper, we recognize this as preliminary data and I myself would not begin to vote for it to be put in the Standards Methods or EPA Methods until I see not only more data from our laboratories but from other laboratories that have checked it out in a field test. This is only the beginning, and if these preliminary results still prove to be promising as others check it out, then I would entertain that idea; but if it doesn't, let's forget it.

Brezenski: During these sessions, we have been using such terms as, attenuated cells, injured cells, damaged cells and a few others that I don't remember at this point. I don't know if anybody has done anything to describe what these terms really mean. I get confused between what is injured and what is dead. Would somebody define these terms for me so that I can get clear in my mind what we are talking about. So far, I have seen no data to show any physiological problems with the cells. There is no enzyme work. No one has shown me a normal physiological reaction taking place within the cell. I would like to see what has happened to the cell as a result of the aquatic environment. If it was in salt water, is some enzymatic system blocked and therefore when it comes into contact with a certain type of medium does it need a specific substrate as a booster? I just fail to see the damage assessment because I haven't seen the proof.

Geldreich: Well, I agree with you. We are assuming many things but we have never proven any of it.

Hufham: We have seen the damage; it results in different types of effects on the cell. You can get lack of separations with chromosomes duplication, in which case you get filaments up to 500 microns long. Unfortunately, you cannot see these as colonies because the width of the cell does not change. This is a stress factor. There have been several studies on this in which they are trying to form filaments in cells and they use heat shock to do it. This is one of the ways of getting cultures synchronized by using heat shock and then bringing them back to the appropriate incubation temperature.

Brezenski: You can see these filamentous forms which Zobel also showed with great hydrostatic pressures. But, as it relates to these studies in terms of your recovery data, and relating back to the reasons why the recoveries were low, we just say they were stressed cells. Zobel showed that when cells are subjected to a certain depth at a certain hydrostatic pressure, the cells formed filamentous branches. We have shown a difference in count assumed on the differences of a stressed cell. But nobody has shown the differences in the stressed cell.

Geldreich: There are various forms of doing this. Zobel used pressure, various people used temperature or antibiotics and we used oxygen. By increasing the percentage of oxygen in the culture you can get the same phenomenon. If you return those to the normal conditions, the cell immediately starts to divide and you get a colony formed. But we have not done it on solid agar. We can see it in the liquid media. Theoretically, if these injured cells are not kept too long and you return the plate to 35 C, you should get a colony formed. I think in the pre-enrichment medium you are getting the micro-colony. Some of these are capable of growing at higher temperatures.

Williams: Getting back to basics, one of the reasons we chlorinate is to attenuate the cells. We like to kill them and I guess my point is that evidently we are making an effort to recover more and more of these attenuated cells and at what point do these attenuated cells no longer have a sanitary significance.

Geldreich: Well, there is some evidence. I think if you remember this morning, Ted, one of the speakers commented about **Salmonella** cultures which had been severely attenuated through a freeze-drying condition and they were proven to be very pathogenic. What we really want is a kill.

Bordner: Ed, you know that we have been investigating chlorinated effluents for sometime. Originally, in some work that a graduate student did with us, we were shocked at the very low levels that were recovered by the MF as compared to the MPN. Most of the results did not fall within, or approach the lower range of the very broad 95% confidence level of the MPN. The MF was that much lower than the confidence levels of the MPN. Also, we have been looking at the M-FC agar for two or more hours at 35 C before placing that same membrane on the same agar at 44.5 C. It looked promising since we knew the interest in the overlay technique.

Geldreich: Are you talking about an overlay or a two layer?

Bordner: A single layer of M-FC is what we were reviewing.

Geldreich: These membranes are sitting on top of the lactose agar which is overlayered on M-FC agar. I thought you meant agar over the membrane.

Bordner: I am talking about the comparison of two-step M-FC and the overlay of the lactose agar over the M-FC agar. In the twenty-two samples of chlorinated effluents and twenty with stream samples, we see very little difference. The recoveries were comparable. I would like to call it temperature acclimation with a ratio of one to one in both cases. There were three samples of chlorinated effluents where there was a background of tiny pinpoint blue colonies. We found them in both the lactose overlay and the non-overlay. It seemed to me that there is some possibility of avoiding the overlay. I am not sure at this point but I think that it would be worth getting more data.

Geldreich: I am intrigued as much as many of you here with this concept of a Millipore membrane that we have heard about today and wonder if that will help too. But I think one of the important things that we must try and get across here is that these are methods that we are only discussing today. They are preliminary and we must have many laboratories field test these things before they go into any published procedures. I don't want any of you to have the illusion that because these three labs did it this is the final work. This is just the beginning and I hope that we evaluate these ideas in many different geographical areas. Many times in the past we personally developed some media that worked great in the Ohio area, but out in the West or in New England it did not work as well, because bacterial flora are different. So whenever we evaluate these procedures it is of great importance that we have as many people involved in it and as many different kinds of samples. We should make our minds up from all of these numbers whether that method goes into the Standard Methods or an EPA manual. Thank you very much.

MEASUREMENT OF FECAL COLIFORMS IN ESTUARINE WATER

by

Alanson P. Stevens, Rosario J. Grasso and John E. Delaney

ABSTRACT

Recoveries of fecal coliform bacteria from estuarine waters were compared using the standard MF procedure and the standard MPN EC Count. The experimental two-step procedure recovered 85% of the MPN count as compared to 24% by the standard MF procedure. Ninety-three percent of the colonies in the experimental procedure did verify.

In later work, significant variation in recoveries using different batches and brands of membrane filters suggested possible influences on the earlier work. Further tests are being conducted.

INTRODUCTION

The fecal coliform concentration in sea water can be measured, at the present time, by the E C MPN procedure, and by a membrane filter method using M-FC broth as the culture medium. However, the inherent disadvantages associated with these procedures have prevented either one from achieving absolute acceptance by marine microbiologists. The multitube method requires a period of three days to complete, and yields a concentration estimate that embodies neither precision nor accuracy. In addition, the MPN procedure is time consuming, requires considerable incubator space, and large amounts of sterilized media and glassware. These requirements restrict the number of samples that can be processed. The direct-count, membrane filter (MF) procedure, on the other hand, although more rapid, possessing greater built-in precision and requiring a minimum of preparatory labor and laboratory glassware, recovers only a fraction of the fecal coliform population in a sea water sample. A study in which over 200 sea water samples were analyzed by both the E C MPN and standard MF procedures indicated that, on the average, only 10% of the fecal coliforms enumerated by the multitube technique were measured by the current MF procedure.

Our studies have indicated that the low recovery exhibited by the membrane filter procedure is intrinsically associated with the immediate exposure of the fecal coliforms to the elevated selective temperature 44.5 C. The stress imposed by this temperature on the individual fecal coliform cells, during the initial 15 minutes of exposure, has been shown to be lethal to the majority of the fecal strains filtered from sea water. Since the poor MF recovery of fecal coliform in sea water is unquestionably related to the immediate exposure of these organisms to the elevated temperature, our initial attempts at developing a more accurate MF test procedure focused on methods of acclimatizing these species before exposing them to the selective temperature.

Enrichment on Minimal Medium at 25 C Methods

It was recognized at the outset, that the medium and the temperature employed during the acclimatization period would have to control the number of bacterial generations, so that subsequent exposure to the selective stage would not foster colony overgrowth. A number of media formulations were tested for their ability to provide optimum resuscitation conditions, while simultaneously controlling growth for the fecal coliforms in sea water samples. Initial studies employed a one per cent (1%) solution of M-FC broth as the minimal medium and full-strength M-FC agar as the growth-indicator medium. This media combination in the two-step, two-day procedure markedly increased the fecal coliform recoveries from sea water, when compared to the results from the standard method one-day, direct count procedure. However, extensive testing of this media combination failed to yield the desired recovery efficiency to warrant its acceptance as a standard test system.

Recent work has focused on formulating and testing a minimal medium composed mainly of simple carbohydrates. It was theorized that these substrates would be easily attacked and metabolized by the fecal coliforms and would foster bacterial cell repair and resuscitation for those that had been attenuated by exposure to sea water. This minimal medium, coupled with full-strength M-FC broth as the selective-growth medium, has yielded very satisfactory recovery levels for fecal coliform in sea water.

The formulation of the minimal medium, labeled L.E.S. Minimal Holding Agar, is as follows:

L.E.S. MINIMAL HOLDING AGAR

Tryptose	0.5 grams/l
Dextrose	0.5 grams/l
Lactose	0.5 grams/l
Oxgall	0.25 grams/l
Sodium Chloride	0.4 grams/l
Agar	15.0 grams/l

L.E.S. Two-step, Two-day Procedure

The minimal holding agar is allowed to warm to room temperature before "prepared" membrane filters are placed on the agar surface. All standard precautions are exercised at this stage to guard against improper placement of the filter on the agar surface. The dishes are tightly sealed and placed into a watertight, plastic bag, making sure that all plates are facing upright. The sealed bags are inverted and incubated in a water bath or air-jacketed incubator regulated at 25 C for 18 ± 2 hours. Subsequent to the enrichment period, the membranes are transferred to absorbent pads in tight sealing petri dishes that have been saturated with Standard Methods M-FC broth. Approximately 1.7 to 1.8 ml of broth are required to saturate an absorbent pad. Excess media should be discarded to prevent excessive and "running" bacterial growth. The dishes are tightly sealed, placed into water-tight plastic bags and incubated in an inverted position in a water bath at 44.5 ± 0.2 C for 24 hours.

Counting of Fecal Coliforms

The fecal coliform colonies are counted with the aid of a stereomicroscope and a light source above, that is approximately perpendicular to the plane of the membrane being counted. The fecal coliform colonies are recognized by their blue coloration and the crystallized deposits on the surface. Both of these identifying characteristics must be employed in counting the fecal coliform colonies. The "quartz" surface appearance has been found to be a complimentary and distinctive characteristic of fecal coliform colonies. Certain non-fecal coliforms capable of growing under the test conditions will form blue-colored colonies but will lack the distinguishing crystalline characteristic on their surface.

RESULTS

Fecal Coliform Recovery – MF vs MPN Procedures

Twenty-five samples of sea water containing varying concentrations of fecal coliform and salt content were examined to determine the recovery efficiency of the L.E.S. two-step, two-day procedure. From each sample, five 10 tube E C MPN analyses were made and ten membrane filters (Millipore HAWG 047S0) were prepared and processed by both the Standard Methods MF procedure and the experimental MF technique. The results, obtained from each of the MF procedures, were logarithmically averaged and proportioned against the average logarithmic MPN result of each sample. Table I presents the percent of recoveries obtained by the two MF procedures for each sample analyzed, and employ the logarithmic average E C MPN result as the true estimate. These data indicate that the two-step, two-day procedure yields a significant recovery increase over that attainable by the Standard Methods MF procedure. The Standard Methods MF procedure produced an average recovery of only 24% while the L.E.S. two-day technique yielded an 86% recovery of the fecal coliform concentration in the sea waters examined. It is worthwhile noting that the lowest recovery obtained by the experimental method (64%) is higher than the highest recovery obtained by the Standard Methods MF procedure (46%). Table I also presents data on the selectivity incorporated into the two-step, two-day MF procedure. Over 93% of the colonies exhibiting the two identifying characteristics of fecal coliform verified as bona fide fecal coliform strains. Overall, the percent verification in the 22 samples ranged from 87 to 100.

Figure I presents a graphical analysis of the recovery data obtained by the E C MPN procedure

TABLE 1. FECAL COLIFORM RECOVERIES BY MEMBRANE FILTER PROCEDURE

Percent Recoveries (*) Standard Methods Procedure (1)	 L.E.S. Procedure (1)	% Verification of L.E.S. Colonies
17	126	92
39	114	95
34	96	88
16	64	93
11	89	96
12	97	95
6	75	100
9	76	90
34	77	95
5	80	91
46	71	87
40	71	100
27	89	95
22	72	91
31	79	92
37	92	92
22	97	----
30	78	96
16	87	94
15	66	96
21	82	91
16	84	87
32	106	100
29	117	90
24	90	100

(*) E C MPN ten tube procedure used as Control.

(1) % Recoveries based on 5 MPNs and 10 MFs per sample.

and the two membrane filter methods. The ratios (Table I) were arrayed in order of magnitude and every other value plotted against the appropriate percentile on log-probability paper. The resulting lines of best fit clearly indicate that a significant difference exists between the recovery attainable by the Standard Methods MF procedure and by the experimental MF method. Based on the 50 percentile (median) ratios, the average recovery of the Standard Methods MF procedure was 22%, while the experimental MF procedure recovered an average of 84% of the fecal coliform concentration in the sea water samples. This analysis indicates that the experimental method, on the average, recovers 2.9 times more fecal coliforms from sea water than the Standard Methods MF procedure. A further analysis of the plots in Figure I clearly demonstrates the improvement in fecal coliform recovery as a result of the two-step, two-day procedure. The plots indicate that the Standard Methods MF procedure recovered less than 19% of the fecal coliform in 40% of the samples and less than 40% in 90% of the samples, while the experimental procedure recovered at least 60% in all samples and less than 72% in only 20% of the samples analyzed.

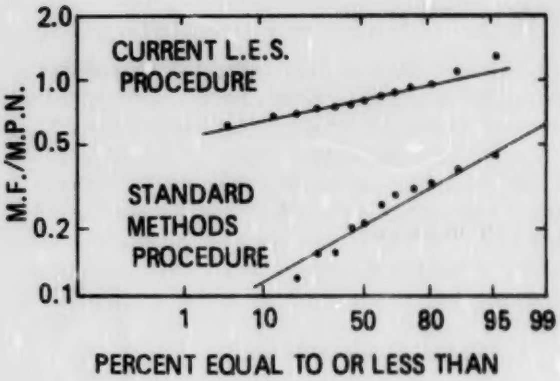

Figure 1.

SUMMARY

The direct count procedure for fecal coliform in estuarine waters, developed under this research project 1, appears to possess the necessary accuracy, precision and selectivity to warrant its acceptance by marine microbiologists as a more than adequate replacement for the E C MPN procedure. The recovery efficiencies of the L.E.S. experimental MF procedure were calculated from MPN values that had not been corrected for their inherent positive bias. If this bias factor had been used in the computation of the recovery ratios, the median recovery percentage (84%) of the experimental procedure would consequently have been adjusted to a more favorable level, namely 92%. This truer recovery capability of the L.E.S. two-step, two-day MF procedure, when coupled with the MF advantages of precision, time of analysis, less preparatory labor and incubator space, are strong arguments for accepting and employing the experimental MF technique as a standard procedure for measuring fecal coliform concentrations in sea water.

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Recent Investigations

Recent studies have indicated that different brands of membrane filters have an effect on the recovery levels of fecal coliform organisms (1, 2). Since all the data generated in developing the L.E.S. two-step, two-day procedure for fecal coliforms was compiled using the Millipore Type HA filter, we deemed it necessary to compare the fecal coliform recoveries attainable by using two brands of membrane filters (Gelman GN-6, lot No. 80706 and Millipore HAWG 047S0, lot No. 934487) in our newly developed method.

This work was not motivated by the desire to prove one membrane filter brand superior over another, but rather by a strong curiosity as to why different reputable brands of filters should produce statistically significant recoveries of fecal coliform. The results of our comparative studies showed that Millipore filters consistently gave lower recoveries than the Gelman filters in the Standard Methods MF procedure for fecal coliform. The Gelman filters, conversely, gave poor recovery compared to the Millipore filters when used in the two-step, two-day L.E.S. procedure. In addition, the Gelman filters produced non-typical fecal coliform colonies subsequent to incubation. These colonies were small with irregular shapes, and varied in color from light brown to the typical fecal coliform blue. It should also be noted, that even with the higher recoveries of fecal coliform by the Gelman filters in the Standard Methods fecal coliform procedure, only 30% of the actual concentrations of fecal coliform in the samples, as judged by the E C MPN method,were recovered.

From these results, it seemed plausible, that any substances present in the membrane filters capable of affecting development of fecal coliform colonies during the incubation period would be soluble in the media, producing pH and other physico-chemical changes. In order to determine the water soluble chemicals in these two filter brands, six filters of each were immersed in 100 ml of distilled water for 18 hours at room temperature, the filters were removed and the following results were obtained upon analysis of the water.

	Gelman	Millipore
	GN-6 Lot #80706	HAWG Lot #93448-7
pH	3.23	6.20
Ammonia-Nitrogen	13.0 mg/l	0.24 mg/l
Ortho-Phosphate (as P)	43 mg/l	0.06 mg/l
Total dissolved solids	185 mg/l	6 mg/l

It was thus possible to project that the differences in quantities of soluble components between brands of filters could somehow be responsible for the differences that were obtained in fecal coliform recoveries. Neither the Standard Methods M-FC broth, nor the two media used in the L.E.S. two-step, two-day procedure have an inorganic buffer system in their formulation.

Low recoveries exhibited by the Gelman filters with the new L.E.S. procedure could be attributed to the increased hydrogen-ion concentration that these filters imposed on the holding-minimal agar, while the higher recoveries by Gelman filters on standard M-FC broth at 44.5 C could be attributed to the leaching of beneficial inorganic nitrogen and phosphate into the medium, as well as the suppressing of the pH change by buffering action of the organics in the formulation. Based on this hypotheses, we added a monopotassium phosphate and dipotassium phosphate buffer system to the L.E.S. minimal holding agar and also to the M-FC broth. Results obtained from experiments employing this buffered media, produced encouraging results with both brands of filters. At present, additional tests are being conducted on estuarine waters using the new buffered media with the two-step, two-day L.E.S. procedure in order to substantiate that either brand of filters can be employed in this technique.

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EVALUATION OF METHODS FOR DETECTING COLIFORMS AND FECAL STREPTOCOCCI IN CHLORINATED SECONDARY SEWAGE EFFLUENTS

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ABSTRACT

Total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) recoveries in chlorinated secondary sewage effluents were investigated using the membrane filter (MF) and multiple-tube (Most probable number, MPN) methods. The LES two-step MF method was found to be comparable to the MPN procedure for determining TC. The TC detection was 1.5 times greater when using the LES two-step technique than that obtained by the M-Endo one-step MF procedure. Fecal coliform recovery by the M-FC MF procedure was lower than the recovery obtained using the MPN method. The use of each of azide-detrose broth, brain-heart infusion broth, and peptone yeast-extract Casitone with the M-Enterococcus agar MF_2 (2-day incubation) procedure was not satisfactory for the recovery of FS. The M-Enterococcus agar procedure with bile broth enrichment (MF_2) or prolonged incubation for 3 days (MF_3) significantly increased FS recovery and were comparable to the MPN method. The results cited should be useful in assessing the efficiency of disinfection practices for waste treatment plants employing effluent chlorination.

INTRODUCTION

The year-round disinfection of wastewater treatment plant effluents has become mandatory in Illinois and in several other states. The most common method of disinfection at treatment plants is chlorination. Its effectiveness has generally been measured by residual chlorine. The Illinois Pollution Control Board (1) requires a limitation on fecal coliforms (FC) densities independent of residual chlorine thus requiring determinations for FC densities in chlorinated effluents. The Board's rules stipulate that fecal coliforms densities in a waste effluent shall not exceed 400/100 ml.

Total coliforms (TC) have been used for measuring the disinfective efficiencies of water and wastewater treatment units. The TC index is still valid and reliable for the water industry. In European countries fecal streptococci (FS) are commonly looked for in the sanitary analysis of water supplies (2). In the United States, they are used currently in conjunction with FC for determining the sanitary quality of water. Although FS determinations are not required by most regulatory agencies the usefulness of the procedure should not be overlooked.

The requirement for bacteria enumeration in treated effluents necessitates the development of adequate and economical procedures for determining bacteria densities in chlorinated effluents. The series of investigations described in this report were undertaken with these objections in mind.

Indicator Organisms. The purpose of the routine bacteriological examination of water samples is usually to estimate the hazard due to fecal pollution and the probability of the presence of pathogenic organisms. The isolation of pathogens from water and sewage is expensive and laborious. It is not a routine practice. Normally occurring bacteria in the intestines of warm-blooded animals have been used as indicators of fecal pollution. Total coliforms, fecal coliforms, and fecal streptococci have all been used as pollution indicators at various times (3, 4). Other bacterial indicators have been proposed. These include **Clostridium, Pseudomonas** and **Aerobacter**. Presently their value has been considered questionable or irrelevant (5).

Correlations between coliforms and pathogenic bacteria have been cited frequently i.e., coliforms vs **Salmonella** (6, 7, 8, 9). Less known is the relationship, if any exist, between coliforms and viruses. A coliform index is not a reliable index for viruses (10, 11). In spite of the lack of documented relationships there is little evidence that enteroviral or other microbial diseases are transmitted frequently by the drinking water route in the absence of coliforms (5).

Until more definitive studies are completed on the relationship of pathogens and indicator organisms, the use of TC for water supplies and FC and FS for sewage and stream quality, as indicators or enteric pollution, are valid.

Bacteria Enumeration. The basic methods for the assay of pollution indicators (TC, FC, and FS) in waters are outlined in **Standard Methods** (4). These include the multiple-tube, or most probable number (MPN) technique and the membrane filter (MF) procedure. **Standard Methods** (4), however, states that "Experience indicates that the MF procedure is applicable to the examination of saline waters but not chlorinated wastewaters". Because the MF technique is not comparable to the MPN procedure and is less time consuming, it seems unfortunate that the MF technique cannot be used as a control procedure by the waste plant operator who uses chlorination.

McKee **et al.** (12) reported on the lack of correlation between MPN and MF techniques while assaying chlorinated settled wastewater for total coliforms. Because monochloramine is the predominant bactericidal agent in chlorinated wastes, they advanced the hypotheses that partial reversibility is responsible for the discrepancy between MPN and MF results; that is, the MF technique produces considerably fewer colonies than the numbers that develop by the MPN method. Presumably, when inactivated cells are deposited on a membrane with limited nutrient availability, the cells cannot rid themselves of monochloramine and therefore cannot grow. However, when inactivated cells are put in an aqueous medium rich in organic matter, such as lactose broth, the monochloramine may diffuse outwardly from the cells, permitting them to recover, grow, and produce gas.

In the McKee **et al.** (12) investigations, the culture media used for the MF technique was the same as that previously described (13). Dehydrated scheduled nutrient (DSN) pads were used. They contained two elements with an upper leaf impregnated with an Endo-type inhibitory nutrient. The results obtained using DSN pads with the MF technique were comparable to those obtained from the confirmed MPN procedures on raw settled wastewater. McCarthy **et al.** (14), though working initially with water, were not satisfied with the one-step, M-Endo broth MF techniques. Their work suggested that enrichment plus an agar substrate (E&A) was superior to the one-step technique based on a higher degree of coliform recovery. Examinations of natural waters and wastewater demonstrated that the E&A results were comparable to standard MPN data. From their work an agar-based medium (LES M-Endo agar) was developed. Its use with the MF technique is basically a two-step enrichment procedure.

The need has developed not only for determining total coliform but also for enumerating fecal coliform densities. Geldreich **et al.** (15) recommended the use of an M-FC medium at incubation temperature of 44.5 ± 0.5 C as part of the MF technique for the direct county of fecal coliform. It has been reported (16, 17, 18) that the determinations for fecal coliform rather than total coliform are a more realistic measurement of the public health significance of microbial discharges in wastewater plants. Illinois requirements specify maximum permissible limits for fecal coliform concentrations in treated effluents. This will require fecal coliform enumeration in chlorinated effluents.

Several investigators (19, 20, 21) reported that **Escherichia coli** injured during a physical or chemical treatment failed to form colonies on membrane filters incubated on M-FC medium, to grow and produce gas in lactose broth or to grow on selected media. Braswell and Hoadley (21) suggested that standard methods for enumeration of total and fecal coliforms in water and wastewater should not be applied to chlorinated effluents.

Even for unchlorinated samples Hufham (22) claimed that a large relative error in the results of MF method was found to be dependent on the brand of MF used, the medium, and the temperature of incubation. A study by Presswood and

Brown (23) showed FC counts incubated on Gelman filters at 44.5 C averaged 2.3 times greater than those on Millipore filters. Hufham (22) suggested that the MF method for FC recovery should not be accepted.

Lattanzi and Mood (24) used the Winter and Sandholzer method for the detection of enterococci. Later Litsky **et al.** (25) suggested the use of glucose azide broth as a presumptive medium and ethyl violet azide (EVA) broth as a confirmatory medium for enterococci detection with MPN procedures.

Slanetz and Bartley (26) proposed the use of M-Enterococcus agar for the isolation of FS by the MF method. Kenner **et al.** (27) introduced the KF streptococcus agar. Rose and Litsky (28) found they could increase the recovery of FS from river water by more than 2-fold when using peptone yeast-extract Casitone (PYC) compared to M-Enterococcus agar. Recently Pavlova **et al.** (29) suggested that fluorescent antibody techniques may be useful, for FS detection, in determining the presence and source of fecal pollution in water.

Objectives

During the course of the study two separate investigations were performed. One dealt principally with TC and FC; the other with FS. The purposes of the study were:

1. To determine whether or not the MF technique for TC, FC and FS detections in chlorinated secondary effluents is comparable to the MPN method.
2. To determine whether or not the LES two-step enrichment MF technique for TC detection, in chlorinated secondary effluents, is comparable to recommended MPN methodology.
3. To develop improvements in the MF method for the detection of FS in chlorinated secondary sewage effluents.

MATERIALS AND METHODS

Grab samples of final settling tank effluents from three wastewater treatment plants serving the cities of Peoria, Morton, and Washington in Illinois were used in the study. A minimum of five effluent samples from each plant were examined. The Peoria plant employs the high-rate activated sludge process treating a combination of domestic and industrial wastewaters. Contact stabilization comparable to the standard-rate activated sludge process is used at Morton. This plant treats principally domestic wastewater. Washington is served by a standard-rate trickling filter plant, treating domestic wastewater also.

One liter portions of each effluent were dosed with calcium hypochlorite (HTH, 70% available chlorine) up through 6 mg/1 of chlorine. The samples were stirred gently but intermittently, and after varying periods of contact (up to 30 min.) they were dechlorinated with an excess of sodium thiosulfate. The dechlorinated samples were assayed immediately for bacterial densities using parallel MPN and MF methods.

The MPN procedures were performed by inoculating a series of four decimal dilutions per sample, using five tubes for each dilution. Lauryl tryptose (LT) broth was used for the presumptive tests in TC and FC determinations. The TC test was confirmed using brilliant green bile (BGB) medium, and was completed with gram-strain. For FC confirmation EC medium at 44.5 ± 0.5 C (water bath) was used. In the MPN procedure for FS tests, axide-dextrose (AD) broth was used for the presumptive test; while ethyl violet azide broth was used for confirmation.

In the MF procedures for TC, FC, FS, three duplications for each sample were filtered through an 0.45 μm membrane filter (Millipore) for each bacterial test. For TC tests, the two-step enrichment for LES M-Endo agar (14) was followed. Occasionally, parallel tests with the standard one-step M-Endo procedure were performed. For TC verification purposes, representative colonies (3 to 6 sheen colonies per filter) were subcultured through LT broth into BGB broth (30). The production of gas on BGB broth was deemed verification.

When using MF procedures for FC detections, the recommendations of Geldreich et al. (16) were followed. Several colonies (3 to 5 blue colonies per filter) grown on the M-FC medium were verified by inoculating in phenol red lactose broth for a 24 to 48 hour period at 35 C and noting gas production. All positive tubes were confirmed at 44.5 C (water bath) in EC broth.

In the determination of FS densities by the MF technique, the standard one-step M-Enterococcus agar (4) was used. According to Sies (31)

M-Enterococcus agar is superior to the KF streptococcus agar for sewage effluents because some of the non-streptococci species in sewage samples grow red and pink colonies on KF Streptococcus agar. The FS counts on the membrane filters were generally made after 2, 3, 4, and 7 days incubation. Parallel tests with the two-step enrichment were also performed. The enrichment media used include AD broth, brain-heart infusion (BHI) broth, bile broth medium (prepared by adding 40 ml sterile 10% oxgall solution to 60 ml sterile BHI broth), and PYC broth. The period of the pre-enrichment was 2-3 hours. For the purpose of FS verification, red and pink colonies (3 to 6 colonies per filter) were fished at random from the membrane filter and inoculated onto a brain-heart infusion agar (BHIA) slant, followed by a catalase test. If the catalase test was negative, then the growth on the BHIA slant was sub-cultured into both a BHI broth and into a bile broth medium for confirmation.

With slight variation all bacteria assay procedures followed **Standard Methods** (4). Generally all the media used were freshly prepared; none of the media used was more than four days old.

RESULTS AND DISCUSSION

Total Coliforms

Multiple-tube versus membrane filter. Consistent with **Standard Methods** (4) recommendations that a comparison be made between MPN and MF techniques before using the MF procedure, a series of bacterial assays on unchlorinated samples from a variety of sources was performed. This evaluation included enumeration for total coliforms as well as fecal coliforms. Table 1 summarizes the results.

TABLE 1. COMPARISON OF THE MPN AND THE MF COLIFORM DENSITIES OF UNCHLORINATED WATERS FROM SEVERAL SOURCES

Source*	Total coliforms/100 ml		Fecal coliforms/100 ml	
	Completed MPN	LES-MF	MPN	MF
Illinois River	1,700	1,400	230	640
Illinois River	1,300	2,000	490	330
Illinois River	790	1,200	170	270
Illinois River	–	–	79	160
Spoon River	2,400	2,700	490	330
Spoon River	3,300	3,100	170	270
Spoon River	460	1,200	140	250
Spoon River	1,300	2,600	790	790
A. S. effluent	92,000,000	80,000,000	35,000,000	19,000,000
A. S. effluent	13,000,000	12,000,000	–	–
A. S. effluent	7,900,000	7,400,000	4,900,000	1,000,000
A. S. effluent	5,400,000	6,700,000	2,400,000	2,100,000
A. S. effluent	–	–	460,000	400,000
A. S. effluent	790,000	1,300,000	33,000	30,000
A. S. effluent	350,000	670,000	79,000	52,000
A. S. effluent	240,000	360,000	79,000	80,000
A. S. effluent	–	–	49,000	59,000
A. S. effluent	–	–	33,000	60,000
T. F. effluent	3,500,000	4,000,000	–	–
T. F. effluent	–	–	490,000	600,000
T. F. effluent	–	–	490,000	410,000
Tert. pond effluent	–	–	790,000	600,000

*A. S. = Activated sludge process
*T. F. = Trickling filter process

Using the paired data t-test technique in testing the hypothesis (H_0) that the mean of the first population is equal to the mean of the second; the results (Tests 1 and 3 of Table 2) do not indicate significant differences in TC and FC recoveries determined by the MPN and MP methods. The comparison for the purposes of this study, therefore, were considered acceptable.

M-Endo (one-step) versus LES M-Endo (two-step). Samples of chlorinated effluents from an activated sludge process were evaluated for TC densities using M-Endo one-step and LES M-Endo agar two-step MF procedures. McCarthy **et al.** (14) performed a similar assessment on unchlorinated water samples from rivers, lakes, and ponds leading to the development of the LES agar-based medium. The results obtained on chlorinated secondary effluents were comparable to those observed by McCarthy **et al.** (14). As shown in Figure 1, the plotted data lie above the equality line, indicating that total coliform recovery by the LES two-step procedure was superior to the M-Endo one-step method. A better development of sheen colonies was also observed on the LES medium.

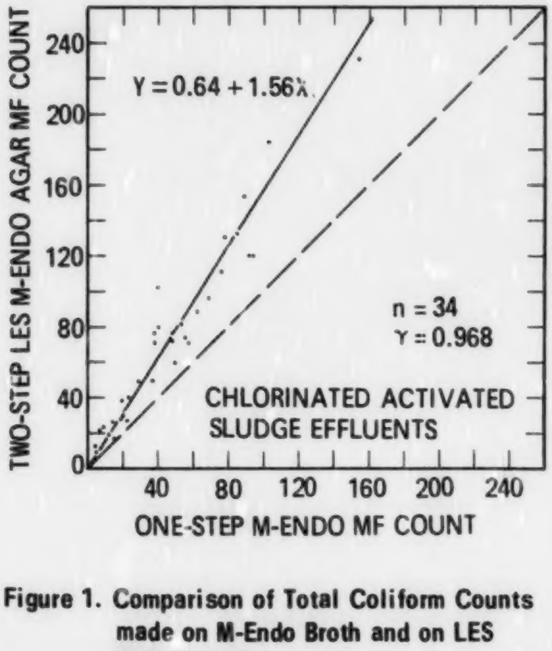

Figure 1. Comparison of Total Coliform Counts made on M-Endo Broth and on LES M-Endo Agar.

The experiences of McCarthy **et al.** (14) and McKee **et al.** (12) were similar with regard to total coliform recovery from unchlorinated wastewater samples. The McCarthy group found no advantage in using an enrichment phase when compared with a one-step agar method on unchlorinated wastes and polluted waters. They suggested that the recovery efficiency for total coliforms was a function of the number of coliforms in the sample and, therefore, in natural waters where smaller numbers of coliforms are likely to exist, an enrichment phase in the MF technique is required, whereas with polluted waters and unchlorinated wastewater the enrichment two-step procedure can be omitted without significant effect on coliform recovery. McKee and his colleagues, however, experienced the lessening of coliform recovery on chlorinated settled wastewater similar to that described for water with a smaller number of coliforms. This suggests that equivalent conditions are encountered when temporarily inactivated colonies exist or a smaller number of colonies are present. In both cases, an enrichment phase would more than likely be required to attain satisfactory coliform recovery using the MF technique.

Although the number of colonies per filter as depicted in Figure 1 exceeded the desirable range of 20 to 80/filter, they were considered satisfactory for comparison purposes. The results correlated well (r = 0.968), and the relationship between the two procedures can be expressed as

$$TC_2 = 0.64 + 1.56\ TC_1$$

where TC_1 and TC_2 are, respectively, the total coliform colonies determined by the one-step and two-steo MF techniques. The total coliform recovery on chlorinated effluents by the LES two-step procedure is about 1.5 times greater than that attained by the M-Endo one-step method.

LES (two-step) versus multiple-tube. The multiple-tube method is considered acceptable for assaying the total coliform densities in chlorinated wastewater effluents. A comparison of the total coliform data resulting from the LES two-step method, which was used in this study, with bacterial densities obtained from parallel multiple-tube observation was therefore pertinent. Using methods described by Thomas (32) the total coliform data for all chlorinated secondary effluents examined are shown in Figures 2 and 3. Figure 2 represents observations of the LES two-step MF technique, and Figure 3 represents observations of the multiple-tube procedure. The figures reflect simply the geometric distribution of the bacterial densities for all effluents using two techniques. Similar curves could have been presented for each type of effluent.

TABLE 2. RESULTS OF THE T-TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN PAIRED OBSERVATIONS

Test number	Compared methods	Bacteria tested	Data used	Number of observation, n	Calculated t	t = 0.05, df*	$H_o:\mu_1=\mu_2$†
1	MPN vs. MF on unchlorinated waters	TC	Table 1	15	0.878	1.761	A
2	MPN vs. LES MF on chlorinated effluents	TC	Figure 4	71	0.343	1.669	A
3	MPN vs. MF on unchlorinated waters	FC	Table 1	20	1.250	1.729	A
4	MPN vs. MF_2 on unchlorinated waters	FS	Table 5	32	0.135	1.696	A
5	MPN vs. MF_2 on chlorinated waters	FS	Figure 6	131	6.5508	1.650	R
6	MPN vs. MF_2 with ADB enrichment	FS	Figure 7	24	4,8911	1.714	R
7	MPN vs. MF_2 with BHIB enrichment	FS	Figure 8	25	0.636	1.711	A
8	MPN vs. MF_2 with bile enrichment	FS	Figure 9	53	11.720	1.676	R
9	MPN vs. MF_2 with PYC enrichment	FS	Figure 10	19	2,724	1.734	R
10	MPN vs. MF_3 on chlorinated effluents	FS	Figure 13	124	0.270	1.658	A

*Tabulated t-distribution with 95% confidence interval for df=n-1 (Reference #41)

†A = accept H_o; R = reject H_o

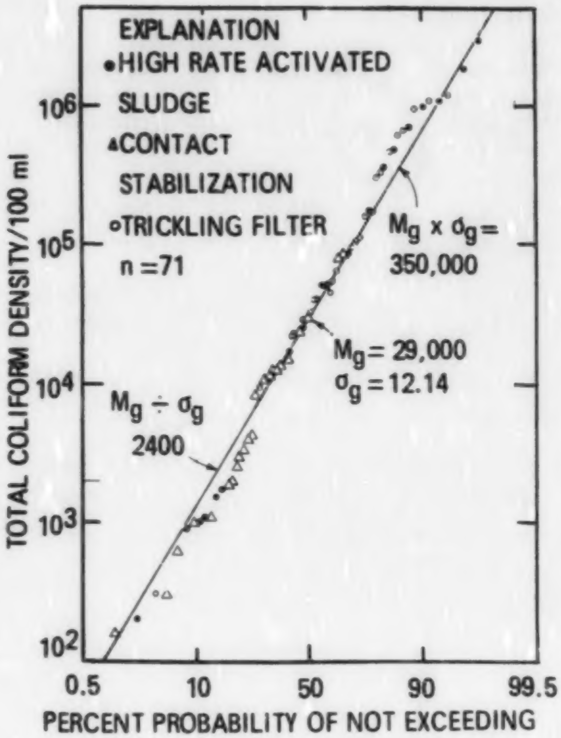

Figure 2. Total Coliform Analysis on LES Two-Step Membrane Filter Samples from Chlorinated Effluents.

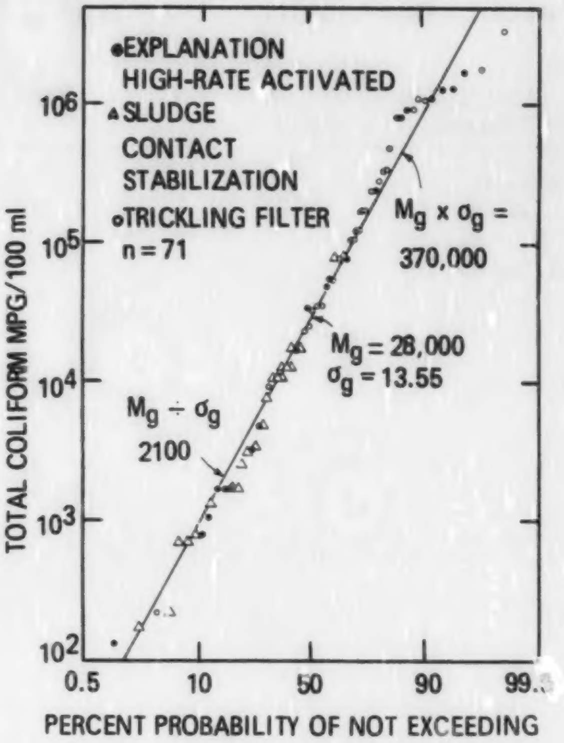

Figure 3. Total Coliform most probable number Analysis made on Chlorinated Effluents

More important for comparative purposes is the summary included in Table 3. For the MF technique, including all data, the geometric mean was 29,000 total coliforms/100 ml; the geometric standard deviation was 12.14; and the arithmetic mean computed from geometric parameters (32) was 650,000/100 ml. Similarly, the MPN data reflected a geometric mean of 28,000 coliforms/ 100 ml, a geometric standard deviation of 13.55; and an arithmetic mean of 700,000/100 ml. All of the data, including that for each type of effluent summarized in Table 2 suggest that the LES two-step MF method for chlorinated effluents is comparable in coliform recovery efficiency to the multiple-tube procedure.

Figure 4 is a graphical presentation for comparative purposes also. For the 71 examples examined, 32 of the MF results are higher and 34 of the MF results are lower than concurrent MPN results. Five observations were found to be identical. The ratios of MF:MPN varied from 0.44 to 5.03 with a median of 1.00.

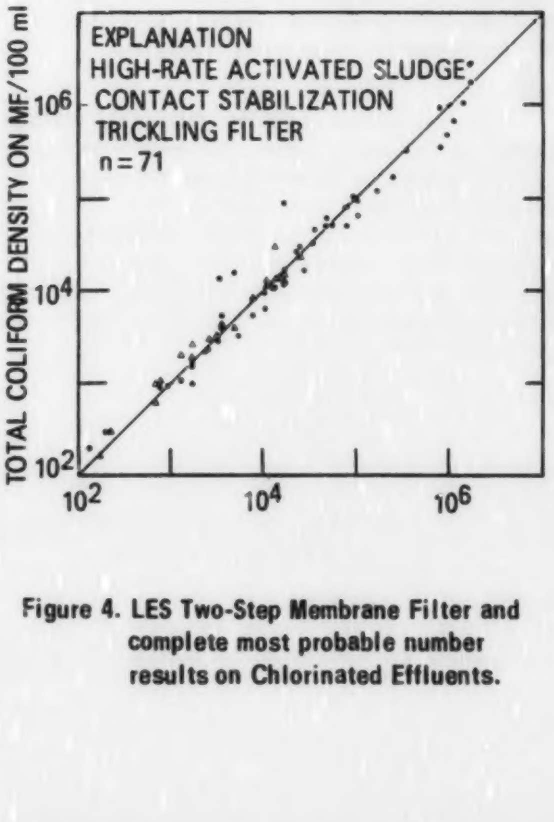

Figure 4. LES Two-Step Membrane Filter and complete most probable number results on Chlorinated Effluents.

TABLE 3. COMPARATIVE RESULTS FOR TOTAL COLIFORM DATA

Chlorinated Effluent	No. of Observations	Test*	Geometric Mean, Per 100 ml	Geometric Standard Deviation	Arithmetic mean †, Per 100 ml
High rate Act. sl.	21	MF	40,000	18.00	2,600,000
		MPN	36,000	20.43	2,900,000
Contact Stabilization	24	MF	5,100	5.16	190,000
		MPN	4,600	5.20	150,000
Trickling Filter	26	MF	110,000	6.31	6,000,000
		MPN	120,000	7.15	7,000,000
Total	71	MF	29,000	12.14	650,000
		MPN	28,000	13.55	700,000

*LES (two-step) MF and completed MPN

†$M = C\, M_g\, \sigma_g\, 1.15 \log \sigma_g$; $C = 1.0$ for MF, $C = 0.851$ for 5-tube MPN

As shown in test 2 of Table 2 the TC recovered by the MPN and LES M-Endo methods are not significantly different. It is concluded that the LES two-step MF technique was as good as the multiple-tube method for assaying total coliforms densities in chlorinated secondary effluents. From the standpoint of time, convenience, freedom from bias, and equipment needs, the LES two-step technique would seem preferable to the multiple-tube technique for chlorinated effluents.

LES (two-step) and multiple-tube verifications. Occasionally, coliform bacteria may fail to reproduce colonies on membrane filters and non-coliform organisms may develop sheen colonies. Verification procedures were undertaken for coliform organisms on all membranes and multiple-tube samples in accordance with procedures described by Geldreich et al. (30). Calculations for verification include:

$$\text{Percent verified (MF)} = \frac{\text{BGB verified sheen colonies}}{\text{total sheen colonies tested}} \times 100$$

$$\text{Percent verified (MPN)} = \frac{\text{Coliform count by the completed test}}{\text{Coliform count by the confirmed test}} \times 100$$

The results of verification for the LES (two-step) MF and confirmed multiple-tube methods are summarized in Table 4. From 263 membrane filters, 1110 sheen colonies were selected for verification: 89.6 percent were verified as coliform organisms. Trickling filter effluent displayed the highest verification (97.5 percent) from the MF technique. It was also the highest (93.3 percent) in using the MPN procedure. From 97 MPN samples a wide range of verifications (22-100 percent) were observed; however, 80 percent of the MPN samples reflected 100 percent verification. The average verifications for both the MF and MPN methods were higher than reported by Geldreich et al. (30): 78.1 percent for MF and 70.3 percent for MPN on samples of natural waters and sewages.

Time effect after dechlorination. During the course of the investigation, the question arose as to whether or not, after the dechlorination of samples, the observed bacterial densities significantly fluctuated with time. This seemed an important consideration because of the time element involved in performing comparative techniques. To investigate this, samples of three types of secondary effluent were chlorinated at varying dosages for a contact time of 15 minutes after which they were dechlorinated as previously described and kept at room temperature (20 to 22 C). Bacterial density assays were undertaken using the LES two-step and the multiple-tube

TABLE 4. VALIDITY OF TWO-STEP MF AND CONFIRMED MPN TESTS

Chlorinated effluent	MF Test			MPN Confirmed Test			
	No. of Membranes	No. of Colonies	Avg. % Verified	Number Tested	100% Verified	% Verified Avg.	% Verified Min.
High rate act. sl.	66	341	86.2	27	22	92.4	46.8
Contact Stabilization	101	393	88.8	35	26	91.0	50.0
Trickling Filter	96	376	97.5	35	30	93.3	22.0
Total	263	1,110	89.6	97	78	92.0	22.0

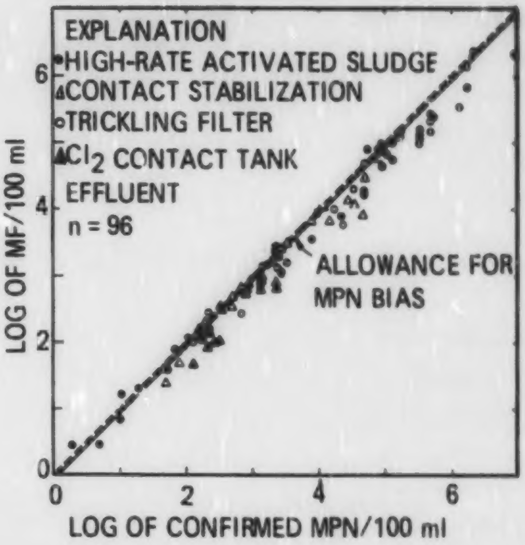

Figure 5. Fecal Coliform densities as determined by the Membrane Filter and most probable number techniques on Chlorinated Effluents.

methods at 15 minute intervals for a 2-hour period. The procedure not only permitted an assessment of the time element but also provided an opportunity for more comparative analyses of the MF versus MPN techniques. The results are summarized in Table 5.

There was no significant change in coliform densities during the more than 2-hour period. A comparison of the paired MF and MPN results indicates the inherent precision of the MF method over that of the MPN.

Fecal Coliforms

Comparison for assaying fecal coliforms was made using the M-FC MF technique recommended by Geldreich et at. (16) and the confirmed MPN procedures (4). These procedures have been accepted for fecal coliform enumerations on unchlorinated wastewater. Four chlorinated effluents were examined. One effluent, representative of the Bloomington-Normal, Ill., Sanitary District's activated sludge process, was collected from a chlorine contact tank effluent stream and immediately dechlorinated; the other three were treated with various dosages of chlorine as previously described.

The results of the two assay methods on the four effluents are shown in Figure 5. It is apparent that most of the plotted points lie below the line of equality. In fact, 78 are below, 12 are above, and 6 are on the line. Adjusting the equality line for MPN bias as described by Thomas (32) does little to change the pattern; 74 points are below and 22 are above the line. In several cases the discrepancy is by a factor of 10 or more which are not apparent in Figure 5.

It can be concluded that the M-FC MF technique for fecal coliform detection, when applied to chlorinated wastewater effluents, is less efficient in recovery than the confirmed MPN procedure. Other media or an enrichment step similar to that used in the LES two-step procedure for total

TABLE 5. COMPARISON OF TOTAL COLIFORM DENSITY (PER 100 ml) IN EFFLUENTS AFTER DECHLORINATION, DETECTED BY MF AND MPN TECHNIQUES

Time (min.) after dechlorination	High rate act. sl. MF	MPN	Contact stabilization MF	MPN	Trickling filter MF	MPN
0	1,500	1,700	6,400	7,900	23,000	23,000
15	1,500	1,700	4,100	6,300	21,000	35,000
30	1,500	1,700	4,000	4,900	25,000	17,000
45	1,600	1,700	4,000	3,300	25,000	28,000
60	1,800	1,700	5,200	3,500	23,000	35,000
75	1,500	2,200	3,200	11,000	24,000	35,000
90	1,600	2,400	3,800	7,900	22,000	49,000
105	1,500	2,400	4,500	3,500	23,000	22,000
120	1,500	2,200	3,900	3,500	23,000	22,000
135	1,500	2,200	–	–	24,000	22,000
Arithmetic mean	1,600	2,000	4,400	5,800	23,000	29,000
Geometric mean	1,500	2,000	4,300	5,200	23,000	27,000
Median	1,500	1,900	4,000	4,900	23,000	25,000
Mode	1,500	1,700	4,000	3,500	23,000	22,000
Coeff. of variation, %	6.9	15.7	21.4	46.8	5.6	33.2
% verified avg.	92.0	96.9	93.5	97.9	91.4	95.3
Range of % verified	77.0-100	69.0-100	83.3-100	78.8-100	73.4-100	53.2-100
Chlorine dosage mg/1	4.0		2.0		3.0	
Contact period, min.	15		15		15	

*MF = LES (two-step)
MPN = Completed tests

coliform might improve the recovery efficiency. Further investigations along these lines would seem justified. Braswell and Hoadley (21) found that the use of Trypticase soy agar was superior to the MPN and MF techniques for E. coli recoveries in chlorinated secondary sewage.

The minimum and maximum fecal coliform ratios of MF/MPN for all tests were 0.17 and 1.46 respectively. The median ratio was 0.70. Based on observations from 96 comparative runs, the relationship of fecal coliform densities in chlorinated effluents for the two procedures can be expressed as

$$\log MF = 0.012 + 0.942 \log MPN$$

The correlation coefficient is 0.987. Until a more precise procedure is developed for using MF techniques in recovering fecal coliforms from chlorinated wastewater, a mathematical expression of this nature may be useful for estimating MPN densities. The results should be multiplied by the factor 0.851 as described by Thomas (32) for an estimate without bias.

Verifications of membrane developed colonies were made using a phenol red lactose broth and EC broth. A total of 616 blue colonies were fished for verification; 87.7 percent were verified. This was lower than 93.2 percent verification reported by Geldreich et al. (16) on pure cultures.

Fecal Streptococci

Multiple-tube versus membrane filter. To compare the MPN and MF techniques a series of FS tests on unchlorinated samples from a variety of sources were performed. The results are summarized in Table 6. A statistical test of the observed data was made using the t-test of pairing observations to determine whether there is a significant difference in FS recoveries by the MPN and MF_2 methods. The results indicate there is no statistical difference in the mean value of the bacterial counts determined by the two methods (test 4 of Table 2). The FS densities obtained from both procedures are comparable and probably have the same sanitary significance. Therefore the laboratory techniques of this study were considered acceptable.

One hundred and thirty-one chlorinated samples taken from three secondary sewage effluents were concurrently assayed for FS densities by the MPN and MF procedures. The colonies developed on the membrane filter were counted after 2,3,4, and 7 days incubation and were designated MF_2, MF_3, MF_4, and MF_7, respectively. The MF_2 and MPN method is recommended by **Standard Methods** (4).

The comparative results of MF_2 and MPN on chlorinated samples are presented graphically in Figure 6. It is apparent that most of the plotted points lie below the line of equality. In fact, 107 plotted points are below, 19 are above, and 5 are on the line. From adjusting the equality line for the MPN bias, as described by Thomas (32), most of the plotted points (97 points) are below the MPN bias reference line, 32 points are above and 2 are on the line. Statistically significant differences were found in FS recoveries, when the MPN procedure with the MF_2 method (Test 5 of Table 2). It can be concluded that the MF_2 procedure gives lower FS recovery on chlorinated effluents than does the MPN technique. It seemed reasonable that enrichment and prolonged incubation might improve FS recovery using the MF method.

Enrichment. Azide dextrose broth is the medium used for the presumptive test of the MPN method for fecal streptococci in waters. Brainheart infusion broth and bile broth medium are the confirmation media of FS for the MF method. These three media were used in this study for enrichment purposes in efforts to enhance FS recovery in chlorinated effluents. The results of FS recovery on M-Enterococcus agars (MF method)

TABLE 6. MOST PROBABLE NUMBER AND MEMBRANE FILTER COUNTS, FECAL |STREPTOCOCCI PER 100 ML IN UNCHLORINATED WATERS

Source	MPN	MF_2*
Illinois River	140	64
	140	60
	130	140
Spoon River	4,600	3,100
	2,200	1,600
	790	900
	700	830
	540	900
	350	300
	280	300
	230	240
	220	200
	170	230
Spring Lake	4	4
Twin Lake	1,300	1,400
	340	420
Havana Farm Pond	110	75
Fiatt Farm Pond	1,400	1,200
High-rate Activated Sludge Process effluent	35,000	34,000
	33,000	30,000
	27,000	30,000
	22,000	26,000
	22,000	24,000
	3,000	2,000
Contact Stabilization Process Effluent	9,400	7,700
	4,900	5,400
	4,900	4,700
	4,600	4,300
Trickling Filter Process Effluent	24,000	24,000
	11,000	13,000
	9,400	7,000
	4,600	5,400

*One-step M-Enterococcus agar MF count with two-day incubation

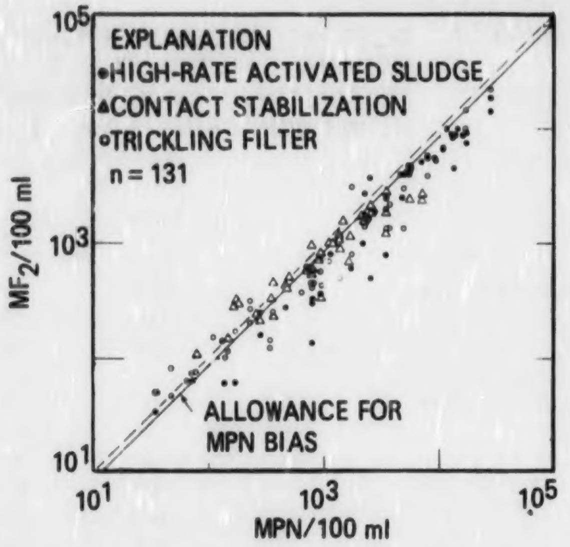

Figure 6. Fecal Streptococci densities as determined by the One-Step Membrane Filter and most probable number technique on Chorinated Effluents.

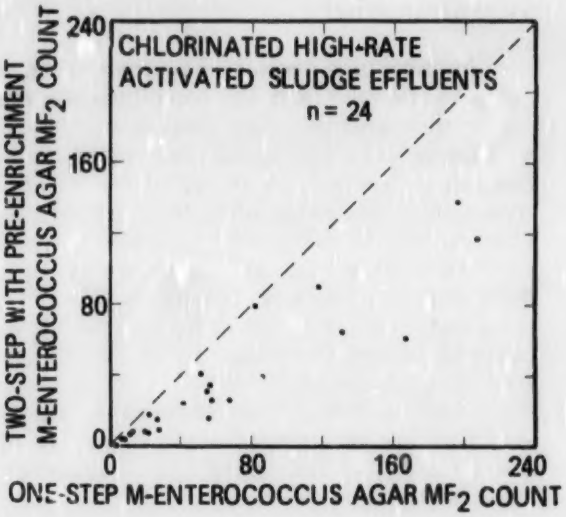

Figure 7. Comparison of Fecal Streptococci Counts on M-Enterococcus Agars with and without Azide Deztrose Broth Enrichment.

with and without enrichment for chlorinated samples are shown in Figure 7, 8, and 9. All FS counts in these figures were made after a two-day incubation. Although the number of colonies per filter as depicted in these figures exceeded the desirable range of 20 to 100 per filter, they were considered satisfactory for comparison purposes.

With AD broth enrichment, all plotted points lie below the equality line (Figure 7). In other words, the FS recovery from chlorinated effluent on M-Enterococcus agar with AD broth enrichment falls far short of that without enrichment. This is substantiated by the t-test (Test 6 of Table 2) and it is concluded therefore that enrichment with AD broth inhibits the FS recovery of chlorinated samples on membranes.

Figure 8 shows no appreciable difference in FS counts with or without BHI broth enrichment. Eleven plotted points lie above, 9 lie below, and 5 points are on equality line. A statistical test (Test 7 of Table 2) suggests no significant difference in FS recoveries from chlorinated effluents determined by the MF method with or without enrichment. Using the least square regression technique, the plotted points in Figure 8 can be fitted as follows:

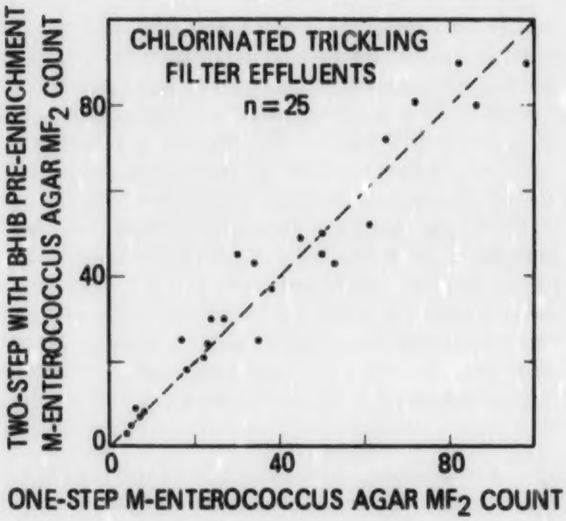

Figure 8. Comparison of Fecal Streptococci Counts on M-Enterococcus Agars with and without Brain.Heart Infusion Broth Enrichment.

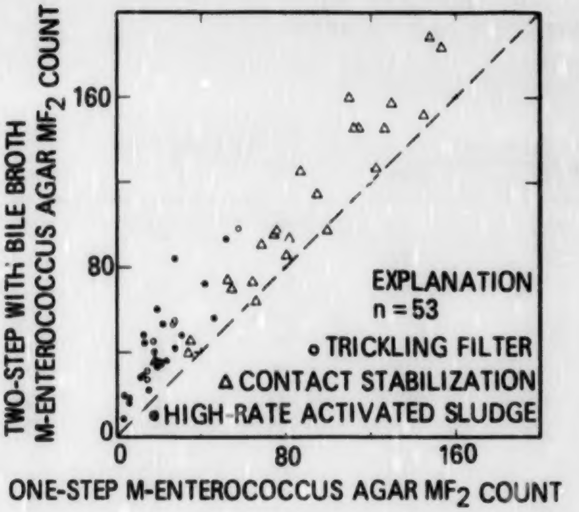

Figure 9. Comparison of Fecal Streptococci Counts of M-Enterococcus Agars with and without Bile Broth Enrichment.

$$Y = 0.097 + 0.98 X$$

in which Y = FS counts by M-Enterococcus agar MF$_2$ with BHI medium enrichment, in organisms per 100 ml; X = FS counts by one-step M-Enterococcus agar MF$_2$ method, in organisms per 100 ml. The correlation coefficient is 0.97. Equation 5 shows the slope to be 0.97 with an intercept of 0.097. Thus the regression line expressed by Equation 1 is almost identical with a 45 degree line. From these tests, it can be reasonably concluded there is no advantage to BHI broth enrichment for the MF method on chlorinated effluent samples.

It is quite evident from the data depicted in Figure 9 that the FS recovery using bile broth enrichment is higher than FS recovery by nonenrichment techniques. Fifty-three comparisons were made on three effluents and only two effluent samples showed the enrichment FS counts slightly less than the nonenrichment. This is confirmed by statistical analyses (Test 8 of Table 2). It is concluded that bile broth enrichment did improve FS recovery on chlorinated effluent samples.

A peptone yeast-extract casitone enrichment broth was suggested by Rose and Litsky (28) for use with the MF method for the enrichment of FS recovery in unchlorinated waters. To determine the efficiency of PYC broth on chlorinated effluents samples, parallel tests were made with PYC broth, bile broth medium, and without enrichment on portions of the same samples.

About one-half of the experimental results were discarded due to extremely high or low counts. The results (68 samples), where filter counts were in the desirable range of 20 to 100, are summarized in Table 7. The values of Table 7 represents a two-day incubation period. For all tested effluents, with few exceptions, the recovery of FS increased with enrichment and especially with bile broth enrichment.

The recovery ratios of enrichment to nonenrichment for each effluent are presented in Table 8. The highest ratios were 2.45:1 and 1.77:1 for bile broth and PYC enrichment, respectively. Similarly, the overall average ratios for the 68 samples were 2.14:1 and 1.60:1. In comparison with the work done by Rose and Litsky (28), the recovery ratio of PYC enrichment to M-Enterococcus agar was 2.44:1 in waters.

Nineteen chlorinated samples were examined for FS densities by both MPN and PYC enrichment MF methods. The results from these assays are depicted in Figure 10. The equality line was adjusted for MPN bias as described by Thomas (32) and used for reference. Fourteen plotted points lie below the equality line, 4 are above and 1 is on the line. A t-test analysis confirmed the differences

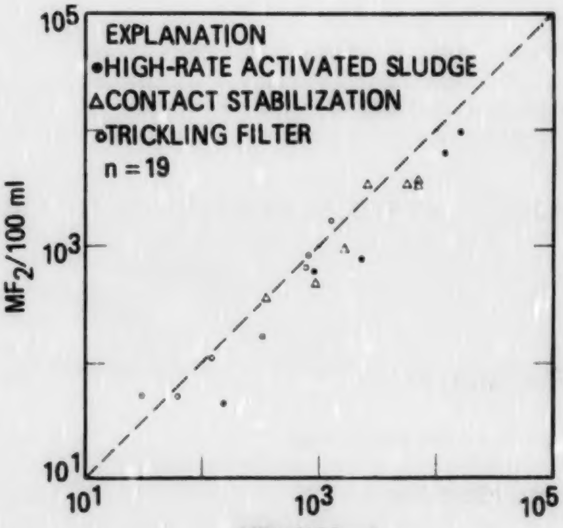

Figure 10. Comparison of Fecal Streptococci densities determined by the MPN and the PYC Enrichment MF methods.

TABLE 7. COMPARISON OF RECOVERY OF FECAL STREPTOCOCCI ON M-ENTEROCOCCUS AGARS WITH AND WITHOUT ENRICHMENT

Sample	Activated Sludge			Contact Stabilization			Trickling Filter		
Number	NE	PYC	Bile	NE	PYC	Bile	NE	PYC	Bile
1	12	22	50	54	75	83	20	35	40
2	75	74	180	28	32	38	16	22	46
3	61	95	160	48	80	90	20	52	50
4	17	30	54	72	95	112	18	34	38
5	32	57	111	61	86	91	26	60	58
6	57	73	97	23	29	29	32	66	90
7	36	47	52	69	91	107	40	80	100
8	19	30	54	44	62	63	60	100	110
9	25	37	68	20	30	30	24	40	54
10	12	20	26	53	104	90	18	29	45
11	29	46	59	59	90	104	60	96	126
12	65	71	90	44	65	80	16	30	38
13	12	22	26	34	36	52	30	38	54
14	17	28	35	88	91	95	20	36	50
15	45	52	74	39	72	62	24	42	48
16	19	30	36	24	34	38	18	32	45
17	31	48	58	53	71	82	11	25	40
18	28	45	48	31	46	70	12	21	39
19	42	66	70	23	38	58	21	22	34
20	52	83	93	40	65	98	50	89	132
21	46	86	96	72	100	122	16	38	36
22							30	36	62
23							18	32	52
24							24	28	56
25							30	56	90
26							16	30	50
Average	35	51	73	47	66	76	26	45	61

Incubation time was 48 hours for all cases; NE means M-Enterococcus agar without enrichment; PYC means with PYC broth enrichment; and Bile means with bile broth medium enrichment.

TABLE 8. FS RECOVERY RATIOS OF ENRICHMENT OF NON-ENRICHMENT

	Ratio of*			
	PYC/NE		Bile/NE	
Chlorinated effluent	Range	Average	Range	Average
High-rate Activated Sludge	0.99-1.87	1.54	1.38-4.16	2.24
Contact Stabilization	1.06-1.96	1.44	1.08-2.45	1.67
Trickling Filter	1.05-2.60	1.77	1.62-3.25	2.45
Overall	0.99-2.60	1.60	1.08-4.16	2.14

* One-step M-Enterococcus agar MF count with two-day incubation

(Test 9 of Table 2). From this test it is concluded that the recovery of FS from chlorinated effluents on PYC enriched membrane filters is less than for the MPN procedure. Although prolonged incubation through seven days on PYC enriched filters showed increasing counts with time. No attempt was made to compare prolonged PYC enriched MF counts with MPN values.

Bile broth enrichment, as mentioned earlier, gave the highest recovery of FS from chlorinated effluent samples. To compare the bile broth enriched MF_2 results with the MPN data, 45 chlorinated samples collected from three sewage effluents were subjected to FS assays, in parallel, by both methods. The results of the analyses are presented in Figure 11. The ratios of the bile enriched MF_2 to the MPN FS densities were calculated, arrayed in order of magnitude, and plotted on log-probability paper. The line of the best fit was drawn. The median, or 50 percentile of the 45 ratios is 1.00. In fact, 4 ratios are equal to, 21 are greater than, and 20 ratios are less than unity. This indicates that the bile enriched MF_2 data is in very close agreement to that data obtained by MPN techniques. It was also observed that there was no significant increase in FS count on the bile enriched filters for prolonged incubation up through seven days. It is concluded that the bile enrichment MF_2 method is superior to the PYC enrichment MF_2 method and comparable to the MPN procedure for the recovery of FS in chlorinated secondary sewage effluents.

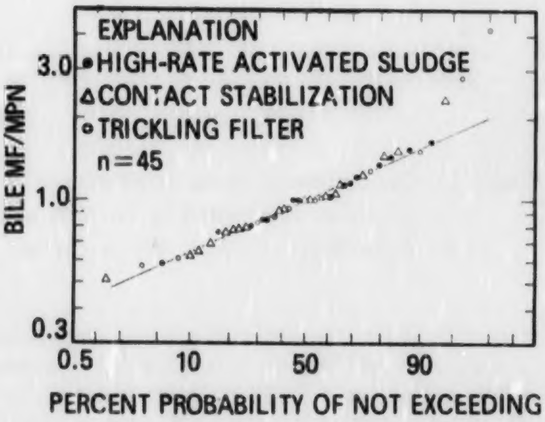

Figure 11. Analysis of Fecal Streptococci Recovery made on Chlorinated Effluents by use of Multipletube (MPN) Test and of M-Enterococcus Agar MF with Bile Broth Enrichment.

Prolonged Incubation. As stated earlier, the M-Enterococcus agar MF_2 (non-enriched) technique tends to produce lower FS recovery than the MPN procedure on chlorinated effluents (see Figure 6). Colonies developed for two-day incubation were generally small. To check the effects of prolonged incubation on FS recovery for the M-Enterococcus agar MF technique all filters were counted at the end of 2, 3, 4, and 7 days incubation periods. Figure 12, a typical example, shows the general trend of the FS counts with incubation time. The FS recovery increased significantly up through the three-day period. After three days, the FS counts leveled off for chlorinated effluents. For the unchlorinated effluent sample, no significant increase was found in FS counts after a two-day incubation. The ratios of MF_3 to MF_2 for unchlorinated and chlorinated effluents are summarized in Table 9. For chlorinated samples the average MF_3/MF_2 values ranged from a low of 1.27 for contact stabilization efflu-

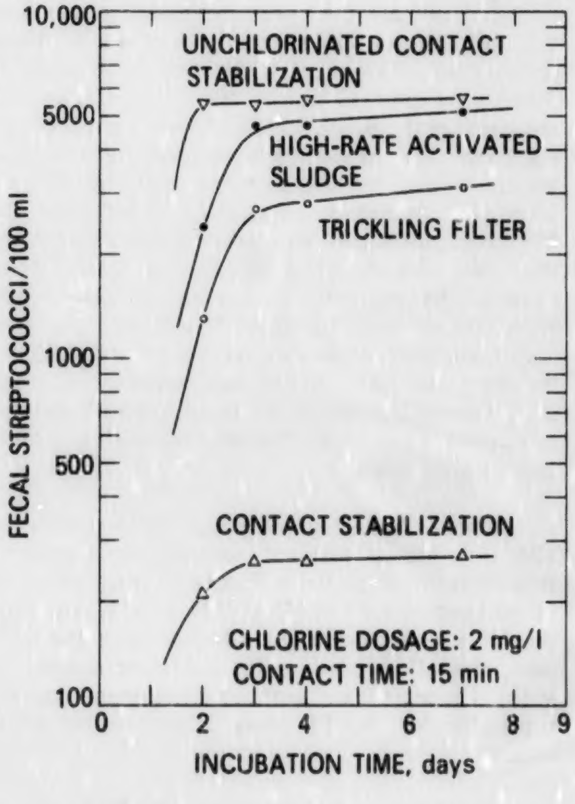

Figure 12. Recovery of Fecal Streptococci on M-Enterococcus Agar from one Unchlorinated and three Chlorinated Secondary Effluents.

TABLE 9. FECAL STREPTOCOCCI COUNT MF_3/MF_2 RATIO

	Unchlorinated			Chlorinated		
Type of Effluent	Number of Sample	Range	Average	Number of Sample	Range	Average
High-rate Activated Sludge	4	1.00-1.33	1.14	41	1.17-4.68	2.12
Contact Stabilization	4	1.00-1.09	1.07	39	1.07-1.67	1.27
Trickling Filter	4	1.00-1.14	1.06	44	1.07-4.28	2.07
Overall	12	1.00-1.33	1.09	124	1.07-4.68	1.84

ent, to a high of 2.12 for high-rate activated sludge with an overall average of 1.84.

To compare the non-enriched MF_3 data with the MPN results, 124 comparisons, made on three chlorinated effluents, are depicted in Figure 13. Seventy-six plotted points are above the line of equality, and 38 are below. Using the corrected MPN bias as a reference line, 101 points are above, 21 are below, and 2 are on the line. The MF_3 results were found to be slightly higher than the MPN data, especially when the FS counts were less than 500/100 ml (Figure 13). For the 124 instances, the geometric mean values were 1,300 MF_3/100 ml and 1,000 MPN/100 ml. The geometric standard deviations were 3.98 and 4.93 for the MF_3 and the MPN methods, respectively. However, a statistical test (Test 10 of Table 2) did not indicate a significant difference between the MPN and MF_3 methods.

When comparing MF_3 and MPN results for 124 chlorinated effluent samples in a manner similar to that depicted in Figure 11 the median, or 50 percentile, for the MF_3/MPN is 1.11. Although the MF_3 values are slightly higher than the MPN data, the MF procedure for 3-day incubation on M-Enterococcus agar, without enrichment, appears applicable for the FS assay of chlorinated effluents.

Verification. A total number of 967 colonies were fished from 306 membrane filters and subjected to the verification procedure outlined in **Standard Methods** (4). The results of the verification are summarized in Table 10. These include colonies grown on filters placed on M-Enterococcus agars with and without enrichment. After two-day incubation, all of 688 colonies isolated from unchlorinated and chlorinated effluents were verified as fecal streptococci. Although Kenner et al. (34) reported similar 100 percent recovery of FS from the membranes for the fecal samples, Rose and Litsky (28) experienced a 94.6 percent FS verification from filters placed on M-Enterococcus agars with and without PYC enrichment for

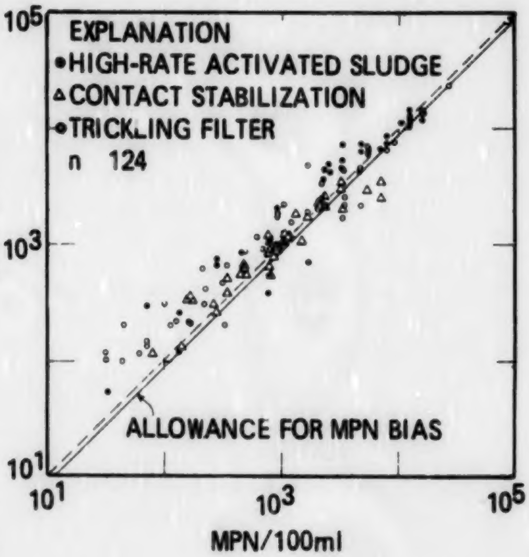

Figure 13. Comparison of Fecal Streptococci densities determined by the MPN and M-Enterococcus Agar MF_3 methods.

TABLE 10. VERIFICATION OF FS GROWN ON FILTERS PLACED ON M-ENTEROCOCCUS AGARS WITH AND WITHOUT ENRICHMENT

Sample	Growth after days of Incubation	No. of Filter	No. of Colonies Examined	Positive Verified	
				No. of Colonies	Percent
Unchlorinated Effluents	2	27	92	92	100
Chlorinated Effluents	2	189	596	596	100
	3	74	234	223	95.3
	4	16	45	42	93.3
Overall	–	306	967	954	98.6

natural waters. From markings placed on the back of petri dishes during this study it was possible to distinguish two, three, and four days growth colonies. About 5-7 percent of the three and four day growth colonies were not verified as FS (Table 10).

SUMMARY AND CONCLUSIONS

Two series of laboratory assays were performed to determine whether or not the standard membrane filter (MF) procedure for total coliforms, fecal coliforms, and fecal streptococci detections on chlorinated secondary sewage effluents was comparable to that obtained by the multiple-tube (MPN) method. If not found to be the case, efforts were made to improve bacteria recoveries using various modifications of the MF method.

Grab samples of secondary effluents were collected with up through 6 mg/1 of chlorine, stirred, and dechlorinated by sodium thiosulfate. After varying periods of contact the samples were assayed for bacteria. Based upon the results derived from this work, the following conclusions were developed.

For chlorinated secondary sewage effluents, the recoveries of TC, FC, and FS by the standard MF (one-step nonenrichment) method is significantly less than that obtained by the standard MPN procedure.

The use of the LES two-step MF method is comparable to the completed MPN procedures for total coliform detection. Total coliform recovery by the LES two-step MF technique is approximately 1.5 times that obtained using the M-Endo, one-step MF procedure. From 273 filters using the LES two-step MF procedure and 1,110 sheen colonies, 89.6 percent were verified as coliform organisms.

Estimates of FC MPN densities may be derived from the MF procedure by using a mathematical relationship similar to $\log \text{MPN} = 1.062 \log \text{MF} - 0.014$. For FC verification, 87.7 percent of 616 blue colonies were verified.

The use of azide-dextrose broth, brain-heart infusion broth and peptone yeast-extract Casitone for enrichment purposes, with the M-Enterococcus agar MF_2 procedure, did not satisfactorily increase the sensitivity of the procedure for FS assays. Enrichment with bile broth medium of the M-Enterococcus agar MF_2 procedure significantly increases the FS recovery to the extent that the procedure is comparable to the multiple-tube method.

The recovery of FS using the membrane filter technique with M-Enterococcus agar increased significantly after three days incubation (MF_3) compared to two days incubation (MF_2); and the MF_3 procedure is comparable to the multiple-tube method for FS detection. The membrane filter technique preferred for FS assays is the MF_2 procedure using M-Enterococcus agar with bile broth enrichment. All of 688 colonies for two-day incubation on filters were verified as fecal streptococci.

ACKNOWLEDGEMENTS

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QUESTION AND ANSWER SESSION

Geldreich: Dr. Lin, with enterococci, we find when we try to go through enrichment with extended incubation time, we run into pediococci and other organisms which are not fecal streps. I don't recall whether you had a table containing this data. Did you check verification when you extended either the time of incubation or the use of these other enrichment devices before you went to M-Enterococcus to show that this increase was still from fecal strep rather than possibly some of these other species that we know will grow on M-Enterococcus or some other strep media? What was that increase, 80% or 90%? That was good.

Lin: Eighty to 90% of the increase was from fecal strep.

Geldreich: So even though you extended the time and you added enrichments you didn't get any more false positives. Good.

Bordner: Dr. Lin, Did you evaluate media other than M-Enterococcus agar for fecal streps, for example PSE agar or the KF medium?

Lin: We restricted our studies to the M-Enterococcus.

Bordner: Shifting back to the M-FC medium, I understood you to say that you haven't looked into enrichment, for fecal coliforms. Is this your next plan?

Lin: You mean this one? Yes, but I didn't succeed.

Bordner: Do you plan to do this in the future?

Lin: Yes.

Bordner: May I ask what brands of filters you usually use?

Lin: Millipore.

Brodsky: Perhaps I should direct this short question to Geldreich. I have read in the literature that M-Enterococcus agar is selective for certain groups or species of streptococci. In comparison with PSE agar and KF agar the terms fecal streptococci and enterococci tend to be used interchangably. Could you clarify these terms?

Geldreich: We find that M-Enterococcus agar is very selective for enterococci but there are other fecal streptococci that we are concerned about. These are from other warm-blooded animals. Feed lots have a tremendous number of streptococci which do not recover too well on M-Enterococcus agar. KF agar and PSE agar recover these species much better. We know we get equivalent results with M-Enterococcus and KF when we use it on domestic sewage because we are looking at enterococci, the sub-group of fecal strep. Since we are working on ratio development of fecal coliform and fecal strep in a stream, there are times when we do have animal feedlot discharges and slaughter house waste. It would be easier to stay with one medium that will recover all of the members of the fecal strep group because otherwise your ratio won't mean a darn thing. We have always recommended the KF agar. Recently PSE agar which is being introduced, looks like an excellent medium. I am sure Warren and Fran have used it and with excellent results. It may be far superior to M-Enterococcus agar, particularly for relationships with fecal coliforms and pollution from feedlots or from domestic wastes.

THE ASTM PROPOSED MEMBRANE FILTER TEST PROCEDURE FOR THE RECOVERY OF FECAL COLIFORMS

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ABSTRACT

Results of a collaborative study of the proposed ASTM test procedure for the recovery of fecal coliforms on membrane filters (MF) were presented. The test procedure is one of a series planned by the ASTM subcommittee D 19.08 .04.02 to evaluate MF materials. Recoveries of fecal coliforms on MF's were compared to those on pour or spread plates using M-FC agar. Analysis of variance indicated differences in filter brands and in the results obtained in different laboratories. The laboratory effect could not be separated from sample effect.

The test procedure did not satisfy all of test objectives and must be rewritten to eliminate variables and options.

INTRODUCTION

Since the acceptance of the membrane filter technique for the isolation and enumeration of total and fecal coliform and fecal streptococci(1), conflicting papers (2,3,4,5,6) have appeared in the literature about the use of membrane filters as a method of evaluating the quality of water. In June of 1973, ASTM brought together microbiologists, membrane filter manufacturers, media manufacturers, regulatory agencies, consumers, and users of membrane filters to discuss the problems that confronted them. At this meeting, it was decided to examine the following parameters of membrane filters:

1. Inhibitory effects
2. Recovery
3. Retention
4. pH
5. Shelf life
6. Sterility

Since existing test procedures for inhibitory effects and recovery were based on 35C (7,8), it was agreed to proceed with a draft for inhibitory effects and recovery using the elevated temperature of 44.5 ± 0.2C for fecal coliforms.

The standard test method for recovery was designed to determine the ability of a membrane to recover fecal coliform organisms from untreated water samples on a selective differential medium. The test method was based on recovery by the M-FC method currently being used in water testing laboratories (1) compared to pour and spread plate procedures.

METHODS AND MATERIALS

Four polluted waters and one raw sewage were collected by each participating laboratory and serially diluted to obtain fecal coliform densities of 20-60 organisms per ml.

Five replicate dilutions of each sample were filtered through test membranes, transferred to M-FC agar and incubated at 44.5 C ± 0.2 C for 22-24 hours. The same dilutions of each sample were also tested on M-FC agar by the pour plate or spread plate technique. The blue colonies were counted with a stereomicroscope for the MF technique and with a Quebec colony counter for the pour and spread plates. Twenty colonies per sample from one representative pour and/or spread plate were verified using lactose broth and EC broth according to the test procedure.

By comparing relicate membrane filter counts with replicate pour and/or spread plate counts, the recovery rate of fecal coliforms on membrane filters was determined. If the arithmetic mean counts on five membrane filters was 85 percent or greather than the arithmetic mean of the five pour and/or spread plate counts, the membrane filter had met the criteria for recovery of fecal coliforms.

A preliminary study of round robin was initiated to determine whether a valid test procedure had been drafted. A common source of media and membrane filters was essential for the study. Six manufacturers supplied membrane filters (0.45 micron pore, white, gridded, sterile 47 mm). Each participating laboratory received the same lot number of membrane filters from the manufacturers. Difco supplied media with the same control numbers to each laboratory.

	Media	Code	Control No.
1.	Bacto-Peptone	0118-01	602509
2.	M-FC Agar	0677-01	586112
3.	Rosolic Acid	3229-09	596061
4.	Lactose Broth	0004-02	597469
5.	EC Medium	0314-02	598803

Several laboratories were able to test all six membranes, whereas, the other labs tested two to four membrane filters. A common data sheet was used to record each laboratory's evaluation. These data sheets were then submitted for statistical analysis to determine whether a valid test procedure had been drafted.

LABORATORIES PARTICIPATING IN PRELIMINARY ROUND ROBIN - COMMITTEE D 19.08 04.02

1. Methods Development and Quality Assurance Research Laboratory
   U. S. Environmental Protection Agency
   Cincinnati, Ohio

2. Canada Center Inland Waters
   Microbiology Laboratory
   Burlington, Ontario

3. Millipore Corporation
   Bedford, Massachusetts

4. Department of Environmental Sciences
   University of Massachusetts
   Amherst, Massachusetts

5. Division of Laboratory Services
   Illinois Environmental Protection Agency
   Chicago, Illinois

6. Gelman Instrument Company
   Ann Arbor, Michigan

7. Sartorius Membrane Filter GMBH
   West Germany

8. Ministry of the Environment
   Division of Laboratories
   Bacteriology Branch
   Rexdale, Ontario

9. Johns-Manville R&D Center
   Denver, Colorado

MEMBRANE MANUFACTURERS OR DISTRIBUTORS PARTICIPATING IN PRELIMINARY ROUND ROBIN TESTS

1.	Sartorius	Beckman Anaheim, California (Manufactured in Germany)
2.	Gelman	Gelman Instrument Company Ann Arbor, Michigan
3.	John-Manville	John-Manville Corporation Denver, Colorado
4.	Oxoid	Med-Ox Chemicals Limited Ottawa, Canada
5.	Millipore	Millipore Corporation Bedford, Massachusetts
6.	S & S	Schleicher and Schuell, Inc. Keene, New Hampshire

RESULTS
(DATA ANALYSIS)

The raw count data from the supplied forms were converted to a machine-readable form and the mean and standard deviations were computed for each set of replicates. These calculated values were then used to determine the recovery of fecal coliform as a percentage of the pour plate (PP) and spread plate (SP) results. In addition, the recovery calculations were repeated, rejecting all counts outside of the ranges of 20 to 60 counts on membrane filters and 30 to 300 colonies on pour or spread plates. These calculations are listed on Table 1. The "ERR" listed is determined from the standard deviations of both measurements by calculating the standard deviations as a percentage of the mean. These percentages were summed and the recovery was multiplied by this percentage to obtain the "ERR." The recovery data was used in the remaining statistical analyses.

The data from all participating laboratories were plotted. In general, the data appeared scattered for all laboratories except one. This laboratory showed no differences between filters.

Data from three laboratories were selected for the analysis of variance. The laboratories were selected on the basis that:

1. All six filters were tested.
2. At least 5 water samples were run.
3. Both spread and pour plate standards were run on all five samples tested.

These laboratories included one filter manufacturer, one university, and one government agency.

Two analyses of variance were run, one using the spread plate standard and one using the pour plate standard. The three variables used in the analyses were:

Laboratories (L)
Filter Membrane (F)
Water Samples (S)

Since the water samples selected by each laboratory were different, the variability due to the samples, the laboratory-sample interaction, and the filter-sample interaction are meaningless for these analyses.

The results of the analysis of variance are summarized in Tables 2 and 3.

The following conclusions can be drawn from the analyses:

1. In both cases the F x L interaction was not statistically significant, i.e., the filters behaved the same in all laboratories.
2. There are differences between filters with manufacturer 3 supplying the best filter and manufacturer 2 supplying the poorest filter. All other filters supplied were about the same. Statistical significance represented the 95 to 97.5 percent level.
3. The difference between laboratories was highly significant (over 99 percent). It is not clear from this analysis if these differences are due to technique in the laboratories or due to differences in water samples used, since these sample differences are included in this effect and in the residual and cannot be separated.

The contribution of the various sources to the total test variance were determined using components of variance analysis for both sets of data. Since the F x L interaction was not significant, a better estimate of residual error can be made by pooling the sum of squares for this term and for the "residual". The calculation of these variances is listed in Tables 4 and 5. It should be noted that the variance contribution of the filters is only 5.5 percent in the case of spread plates or 7.3 percent in the case of the pour plates of the total variance. The large sources of variance are the "residual" (unexplained or random) variance and the inter-laboratory differences.

The net effect of these large variances is to render the results of the test suspect. The expected value of the test of any filter by any laboratory would fall in a range of ± 2 standard deviations 95 percent of the time. This factor is ± 78 for spread plates and ± 85 for pour plates. This variability makes the proposed test useless.

The proposed test procedure permitted the use of either spread or pour plates as the standard at the discretion of the testing laboratory. A scatter plot of the results obtained in this test from spread and pour plates on the same samples from all laboratories is shown in Figure 1, although higher counts were obtained using spread plates. Statistical analysis of the data indicated that the variability of the data from the two methods was not statistically significant.

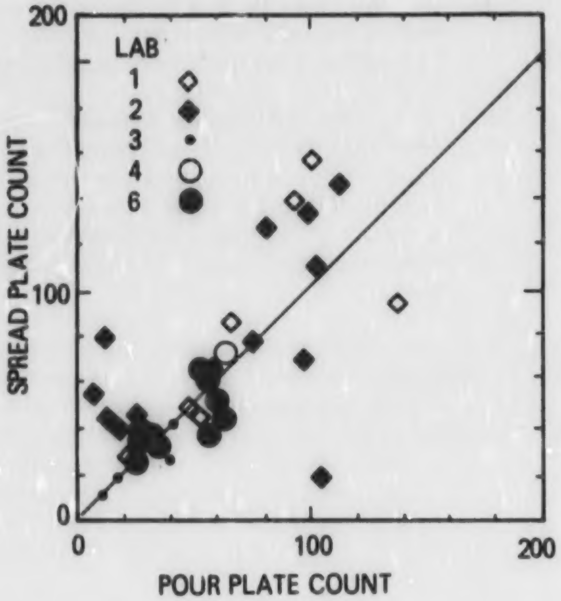

Figure 1. Scatter Plot of Spread and Pour Plate Results.

CONCLUSIONS

We can reach the following conclusions based on the data analyses:

1. There are differences between filters.
2. Different results are reached in different laboratories although filters behave similarly in all laboratories.
3. Unknown causes contributed markedly to the variability of test results. This could be due to differences in bacterial population and pollutants in the test samples.
4. There is no evidence that pour or spread plates are more variated. Spread plates have a higher recovery than pour plates.
5. We do not have a satisfactory test.

RECOMMENDATIONS

The test as presently set up is not satisfactory. Since we cannot separate the effects of samples from the effect of laboratories with the present data, we cannot be sure if the variability is due to differences between technique or to sample differences. If the same samples could be run at a limited number of laboratories with a limited number of filters, these effects could be clarified. This would require either a mixed pure culture approach or the shipping of samples by air from a common point with all its inherent problems. Neither proposal is ideal.

The procedure should be rewritten to leave nothing to the discretion of the person running the test before another round robin is proposed. A decision should be made limiting the standard to either spread or pour plates to reduce the amount of work required.

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Table I. Fecal Coliform Recovery Data

L A B	H2O SPL TYP	M F G	S T D	T S T	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	 SELECTED DATA AVG	 ERR
1	S-1	1	GA	PP	92.92	57.32	83.33	28.46
1	S-1	1	GA	SP	80.15	43.27	88.49	27.87
1	S-1	1	PA	PP	46.01	48.66		
1	S-1	1	PA	SP	39.69	38.92		
1	S-1	2	GA	PP	12.38	9.97		
1	S-1	2	GA	SP	10.68	7.77		
1	S-1	2	PA	PP	77.87	68.70		
1	S-1	2	PA	SP	67.17	54.08		
1	S-1	3	GA	PP	114.15	54.80	86.00	32.38
1	S-1	3	GA	SP	98.47	39.68	91.32	31.96
1	S-1	3	PA	PP	139.82	113.48	105.33	74.58
1	S-1	3	PA	SP	120.61	88.59	111.85	76.23
1	S-1	4	GA	PP	69.91	65.86		
1	S-1	4	GA	SP	60.30	52.16		
1	S-1	5	GA	PP	111.50	49.28	84.00	28.42
1	S-1	5	GA	SP	96.18	35.10	89.20	27.82
1	S-1	5	PA	PP	107.07	66.56	87.30	40.13
1	S-1	5	PA	SP	92.36	50.30	92.92	40.15
1	S-1	6	GA	PP	69.02	54.04	71.66	19.24
1	S-1	6	GA	SP	59.54	42.54	76.10	18.42
1	S-1	6	PA	PP	109.73	98.65	104.44	72.84
1	S-1	6	PA	SP	94.65	77.80	110.91	74.41
1	S-2	1	GA	PP	42.94	20.14	35.79	10.13
1	S-2	1	GA	SP	29.13	15.84		
1	S-2	1	PA	PP	33.76	19.64	39.52	16.14
1	S-2	1	PA	SP	22.89	15.03		
1	S-2	2	GA	PP	3.63	1.18		
1	S-2	2	GA	SP	2.46	0.99		
1	S-2	2	PA	PP	32.47	10.95	35.52	8.83
1	S-2	2	PA	SP	22.02	9.08		
1	S-2	3	GA	PP	66.66	10.36	61.43	6.22
1	S-2	3	GA	SP	45.21	10.42		
1	S-2	3	PA	PP	60.68	15.86	53.41	11.14
1	S-2	3	PA	SP	41.15	13.84		
1	S-2	4	GA	PP	36.53	18.73	41.39	16.82
1	S-2	4	GA	SP	24.78	14.56		
1	S-2	5	GA	PP	48.07	23.60	39.79	12.74
1	S-2	5	GA	SP	32.60	18.45		
1	S-2	5	PA	PP	58.76	21.02	44.33	24.76
1	S-2	5	PA	SP	39.85	17.25		

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

TST/STD COLUMNS: GA = GRIDDED MEMBRANE ON AGAR
PA = PLAIN MEMBRANE ON AGAR
SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	RECOVERY - PERCENT OF STD			
					ALL DATA		SELECTED DATA	
					AVG	ERR	AVG	ERR
1	S-2	6	GA	PP	9.40	4.70		
1	S-2	6	GA	SP	6.37	3.66		
1	S-2	6	PA	PP	33.76	11.25	33.76	11.25
1	S-2	6	PA	SP	22.89	9.35		
1	P-1	1	GA	PP	68.55	45.89		
1	P-1	1	GA	SP	68.55	24.87		
1	P-1	1	PA	PP	44.92	47.50	39.64	14.72
1	P-1	1	PA	SP	44.92	33.72		
1	P-1	2	GA	PP	44.63	27.25	58.57	5.84
1	P-1	2	GA	SP	44.63	13.56		
1	P-1	2	PA	PP	51.59	34.81		
1	P-1	2	PA	SP	51.59	18.99		
1	P-1	3	GA	PP	74.20	44.84		
1	P-1	3	GA	SP	74.20	22.09		
1	P-1	3	PA	PP	76.66	47.81		
1	P-1	3	PA	SP	76.66	24.30		
1	P-1	4	GA	PP	50.72	37.12		
1	P-1	4	GA	SP	50.72	21.57		
1	P-1	5	GA	PP	62.17	37.32		
1	P-1	5	GA	SP	62.17	18.25		
1	P-1	5	PA	PP	65.21	46.16		
1	P-1	5	PA	SP	65.21	26.16		
1	P-1	6	GA	PP	50.00	40.26	53.57	12.35
1	P-1	6	GA	SP	50.00	24.93		
1	P-1	6	PA	PP	62.60	37.76		
1	P-1	6	PA	SP	62.60	18.56		
1	P-2	1	GA	PP	62.61	22.14	62.61	22.14
1	P-2	1	GA	SP	62.33	21.82	62.33	21.82
1	P-2	1	PA	PP	72.52	30.30	72.52	30.30
1	P-2	1	PA	SP	72.19	29.91	72.19	29.91
1	P-2	2	GA	PP	56.75	17.57	56.75	17.57
1	P-2	2	GA	SP	56.50	17.29	56.50	17.29
1	P-2	2	PA	PP	68.46	22.76	68.46	22.76
1	P-2	2	PA	SP	68.16	22.42	68.16	22.42
1	P-2	3	GA	PP	119.81	61.68	103.60	37.01
1	P-2	3	GA	SP	119.28	60.97	103.13	36.48
1	P-2	3	PA	PP	90.99	29.73	90.99	29.73
1	P-2	3	PA	SP	90.58	29.28	90.58	29.28
1	P-2	4	GA	PP	72.97	26.86	72.97	26.86

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

TST/STD COLUMNS: GA = GRIDDED MEMBRANE ON AGAR
PA = PLAIN MEMBRANE ON AGAR
SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	*	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	* * *	 SELECTED DATA AVG	 ERR	* * *
1	P-2	4	GA	SP	*	72.64	26.48	*	72.64	26.48	*
1	P-2	5	GA	PP	*	79.72	25.71	*	79.72	25.71	*
1	P-2	5	GA	SP	*	79.37	25.31	*	79.37	25.31	*
1	P-2	5	PA	PP	*	90.99	26.02	*	90.99	26.02	*
1	P-2	5	PA	SP	*	90.58	25.58	*	90.58	25.58	*
1	P-2	6	GA	PP	*	44.14	15.44	*	48.79	13.77	*
1	P-2	6	GA	SP	*	43.94	15.21	*	48.57	13.53	*
1	P-2	6	PA	PP	*	71.17	25.84	*	71.17	25.84	*
1	P-2	6	PA	SP	*	70.85	25.47	*	70.85	25.47	*
1	P-3	1	GA	PP	*	110.06	51.02	*	89.17	17.41	*
1	P-3	1	GA	SP	*	84.69	43.29	*	75.97	10.20	*
1	P-3	1	PA	PP	*	84.75	26.66	*	75.71	18.38	*
1	P-3	1	PA	SP	*	65.21	23.62	*	64.50	11.73	*
1	P-3	2	GA	PP	*	79.26	23.96	*	74.69	17.58	*
1	P-3	2	GA	SP	*	60.99	21.34	*	63.63	11.09	*
1	P-3	2	PA	PP	*	100.00	35.79	*	80.03	15.95	*
1	P-3	2	PA	SP	*	76.95	31.20	*	68.18	9.44	*
1	P-3	3	GA	PP	*	110.36	28.46	*			*
1	P-3	3	GA	SP	*	84.92	25.94	*			*
1	P-3	3	PA	PP	*	114.32	27.63	*			*
1	P-3	3	PA	SP	*	87.97	25.45	*			*
1	P-3	4	GA	PP	*	80.18	35.63	*	72.02	27.41	*
1	P-3	4	GA	SP	*	61.70	30.35	*	61.36	19.61	*
1	P-3	5	GA	PP	*	92.68	26.11	*	81.55	14.04	*
1	P-3	5	GA	SP	*	71.31	23.49	*	69.48	7.73	*
1	P-3	5	PA	PP	*	90.24	22.37	*	87.27	19.06	*
1	P-3	5	PA	SP	*	69.44	20.52	*	74.35	11.71	*
1	P-3	6	GA	PP	*	65.54	27.39	*	65.54	27.39	*
1	P-3	6	GA	SP	*	50.43	23.46	*	55.84	19.93	*
1	P-3	6	PA	PP	*	96.95	23.87	*			*
1	P-3	6	PA	SP	*	74.60	21.92	*			*
1	P-4	1	GA	PP	*	37.32	14.88	*	44.70	10.90	*
1	P-4	1	GA	SP	*	25.27	8.01	*			*
1	P-4	1	PA	PP	*	38.52	16.74	*	46.13	12.90	*
1	P-4	1	PA	SP	*	26.08	9.20	*			*
1	P-4	2	GA	PP	*	5.19	9.26	*			*
1	P-4	2	GA	SP	*	4.18	5.93	*			*
1	P-4	2	PA	PP	*	42.71	21.57	*	51.15	17.91	*
1	P-4	2	PA	SP	*	28.91	12.24	*			*

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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Table I. Fecal Coliform Recovery Data (Cont'd.)

L	H2O	M	S	T	RECOVERY - PERCENT OF STD			
A	SPL	F	T	S	ALL DATA		SELECTED DATA	
B	TYP	G	D	T	AVG	ERR	AVG	ERR
1	P-4	3	GA	PP	52.49	19.18	62.86	13.23
1	P-4	3	GA	SP	35.54	10.08		
1	P-4	3	PA	PP	50.89	19.56	60.95	13.98
1	P-4	3	PA	SP	34.45	10.43		
1	P-4	4	GA	PP	43.51	15.66	52.11	10.68
1	P-4	4	GA	SP	29.45	8.19		
1	P-4	5	GA	PP	48.90	23.84	54.68	17.52
1	P-4	5	GA	SP	33.10	13.43		
1	P-4	6	GA	PP	33.33	17.32	39.92	14.56
1	P-4	6	GA	SP	22.56	9.88		
1	P-4	6	PA	PP	35.92	22.80	50.79	12.36
1	P-4	6	PA	SP	25.00	13.39		
1	P-5	1	GA	PP	88.50	25.21		
1	P-5	1	GA	SP	31.21	11.47		
1	P-5	1	PA	PP	34.09	25.33		
1	P-5	1	PA	SP	12.02	9.93		
1	P-5	2	GA	PP	24.52	10.54		
1	P-5	2	GA	SP	8.64	4.43		
1	P-5	2	PA	PP	115.70	17.79		
1	P-5	2	PA	SP	40.81	9.64		
1	P-5	3	GA	PP	125.28	34.37		
1	P-5	3	GA	SP	44.18	15.77		
1	P-5	3	PA	PP	111.11	38.37		
1	P-5	3	PA	SP	39.18	16.77		
1	P-5	4	GA	PP	87.73	22.77		
1	P-5	4	GA	SP	30.94	10.58		
1	P-5	5	GA	PP	102.29	23.55		
1	P-5	5	GA	SP	36.08	11.28		
1	P-5	5	PA	PP	109.19	28.59		
1	P-5	5	PA	SP	38.51	13.26		
1	P-5	6	GA	PP	76.24	28.38		
1	P-5	6	GA	SP	26.89	12.23		
1	P-5	6	PA	PP	62.45	222.23		
1	P-5	6	PA	SP	22.02	80.20		
2	S-2	1	GA	PP	152.00	30.00		
2	S-2	1	GA	SP	145.01	29.33		
2	S-2	1	PA	PP	117.06	44.56		

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	RECOVERY - PERCENT OF STD			
					ALL DATA		SELECTED DATA	
					AVG	ERR	AVG	ERR
2	S-2	1	PA	SP	111.68	43.06		
2	S-2	3	GA	PP	135.59	21.69		
2	S-2	3	GA	SP	125.13	27.16		
2	S-2	3	PA	PP	120.05	40.30		
2	S-2	3	PA	SP	110.79	43.52		
2	S-2	6	GA	PP	100.69	41.54		
2	S-2	6	GA	SP	79.65	40.01		
2	S-2	6	PA	PP	120.25	28.26		
2	S-2	6	PA	SP	95.12	30.89		
2	P-1	1	GA	PP	179.60	88.65		
2	P-1	1	GA	SP	113.88	29.43		
2	P-1	1	PA	PP	154.97	122.02		
2	P-1	1	PA	SP	98.26	54.26		
2	P-1	3	GA	PP	182.47	111.64		
2	P-1	3	GA	SP	138.57	44.75		
2	P-1	3	PA	PP	167.43	90.64		
2	P-1	3	PA	SP	127.15	32.10		
2	P-1	6	GA	PP	301.30	152.02		
2	P-1	6	GA	SP	298.96	69.88		
2	P-1	6	PA	PP	258.11	107.87		
2	P-1	6	PA	SP	256.10	37.68		
2	P-2	1	GA	PP	164.48	84.18		
2	P-2	1	GA	SP	147.22	87.21		
2	P-2	1	PA	PP	156.55	102.77		
2	P-2	1	PA	SP	140.12	103.28		
2	P-2	3	GA	PP	271.84	202.68		
2	P-2	3	GA	SP	251.74	109.13		
2	P-2	3	PA	PP	101.55	80.43		
2	P-2	3	PA	SP	94.04	45.13		
2	P-2	6	GA	PP	190.08	95.81		
2	P-2	6	GA	SP	165.00	37.27		
2	P-2	6	PA	PP	205.50	131.25		
2	P-2	6	PA	SP	178.39	64.31		
2	P-3	1	GA	PP	764.70	433.57		
2	P-3	1	GA	SP	126.21	69.66		
2	P-3	1	PA	PP	749.01	369.43		
2	P-3	1	PA	SP	123.62	59.12		
2	P-3	3	GA	PP	1275.86	922.44		

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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Table I. Fecal Coliform Recovery Data (Cont'd.)

LAB	H2O SPL TYP	MFG	STD	TST	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	 SELECTED DATA AVG	 ERR
2	P-3	3	GA	SP	187.81	55.91		
2	P-3	3	PA	PP	1117.24	856.65		
2	P-3	3	PA	SP	164.46	56.15		
2	P-3	6	GA	PP	1516.27	1196.57		
2	P-3	6	GA	SP	237.09	115.82		
2	P-3	6	PA	PP	1530.23	1221.86		
2	P-3	6	PA	SP	239.27	119.12		
2	P-4	1	GA	PP	391.86	136.18		
2	P-4	1	GA	SP	158.96	88.53	113.26	30.49
2	P-4	1	PA	PP	117.44	127.21		
2	P-4	1	PA	SP	47.64	61.58		
2	P-4	3	GA	PP	447.56	426.09	196.55	52.80
2	P-4	3	GA	SP	169.90	137.35	113.43	55.21
2	P-4	3	PA	PP	840.24	703.96		
2	P-4	3	PA	SP	318.98	221.43		
2	P-4	6	GA	PP	375.18	439.85		
2	P-4	6	GA	SP	248.30	98.97		
2	P-4	6	PA	PP	349.63	507.80		
2	P-4	6	PA	SP	231.40	157.03		
3	S-1	1	GA	PP	83.60	41.21		
3	S-1	1	GA	SP	92.72	55.67		
3	S-1	2	GA	PP	95.08	28.70		
3	S-1	2	GA	SP	105.45	43.16		
3	S-1	3	GA	PP	96.72	29.91		
3	S-1	3	GA	SP	107.27	44.69		
3	S-1	4	GA	PP	50.81	20.17		
3	S-1	4	GA	SP	56.36	28.42		
3	S-1	5	GA	PP	68.85	23.28		
3	S-1	5	GA	SP	76.36	34.02		
3	S-1	6	GA	PP	227.86	132.46		
3	S-1	6	GA	SP	252.72	174.04		
3	S-2	1	GA	PP	92.07	38.19	92.07	38.19
3	S-2	1	GA	SP	88.15	35.26	88.15	35.26
3	S-2	2	GA	PP	107.92	30.57	107.92	30.57
3	S-2	2	GA	SP	103.31	27.75	103.31	27.75
3	S-2	3	GA	PP	118.81	36.56	118.81	36.56

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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Table I. Fecal Coliform Recovery Data (Cont'd.)

L	H2O	M	S	T	*	RECOVERY - PERCENT OF STD					*
A	SPL	F	T	S	*	ALL DATA		*	SELECTED DATA		*
B	TYP	G	D	T	*	AVG	ERR	*	AVG	ERR	*
3	S-2	3	GA	SP	*	113.74	33.33	*	113.74	33.33	*
3	S-2	4	GA	PP	*	103.46	50.23	*	103.46	50.23	*
3	S-2	4	GA	SP	*	99.05	46.63	*	99.05	46.63	*
3	S-2	5	GA	PP	*	103.46	50.23	*	103.46	50.23	*
3	S-2	5	GA	SP	*	99.05	46.63	*	99.05	46.63	*
3	S-2	6	GA	PP	*	82.17	31.84	*	82.17	31.84	*
3	S-2	6	GA	SP	*	78.67	29.32	*	78.67	29.32	*
3	P-1	1	GA	PP	*	114.59	39.13	*	96.56	14.28	*
3	P-1	1	GA	SP	*	122.47	45.54	*	103.21	18.40	*
3	P-1	2	GA	PP	*	87.12	32.22	*	87.12	32.22	*
3	P-1	2	GA	SP	*	93.11	37.27	*	93.11	37.27	*
3	P-1	3	GA	PP	*	102.14	31.33	*	102.14	31.33	*
3	P-1	3	GA	SP	*	109.17	36.80	*	109.17	36.80	*
3	P-1	4	GA	PP	*	83.69	32.92	*	83.69	32.92	*
3	P-1	4	GA	SP	*	89.44	37.90	*	89.44	37.90	*
3	P-1	5	GA	PP	*	88.84	37.90	*	88.84	37.90	*
3	P-1	5	GA	SP	*	94.95	43.40	*	94.95	43.40	*
3	P-1	6	GA	PP	*	109.01	36.98	*	103.00	34.89	*
3	P-1	6	GA	SP	*	116.51	43.06	*	110.09	40.64	*
3	P-2	1	GA	PP	*	76.74	17.20	*			*
3	P-2	1	GA	SP	*	72.52	19.20	*			*
3	P-2	2	GA	PP	*	103.48	26.05	*			*
3	P-2	2	GA	SP	*	97.80	28.59	*			*
3	P-2	3	GA	PP	*	111.62	22.03	*			*
3	P-2	3	GA	SP	*	105.49	25.11	*	99.18	6.23	*
3	P-2	4	GA	PP	*	73.25	19.52	*			*
3	P-2	4	GA	SP	*	69.23	21.26	*			*
3	P-2	5	GA	PP	*	89.53	13.19	*			*
3	P-2	5	GA	SP	*	84.61	15.91	*			*
3	P-2	6	GA	PP	*	80.23	22.23	*			*
3	P-2	6	GA	SP	*	75.82	24.09	*			*
3	P-3	1	GA	PP	*	107.65	45.04	*	107.65	45.04	*
3	P-3	1	GA	SP	*	161.06	24.86	*	161.06	24.86	*
3	P-3	2	GA	PP	*	106.63	52.29	*	106.63	52.29	*
3	P-3	2	GA	SP	*	159.54	36.12	*	159.54	36.12	*
3	P-3	3	GA	PP	*	119.89	64.64	*	110.96	53.86	*
3	P-3	3	GA	SP	*	179.38	49.35	*	166.03	36.74	*
3	P-3	4	GA	PP	*	79.08	41.79	*	79.08	41.79	*

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

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L   H2O   M   S    T    *        RECOVERY - PERCENT OF STD           *
A   SPL   F   T    S    *      ALL DATA       *   SELECTED DATA      *
B   TYP   G   D    T    *     AVG       ERR   *    AVG       ERR     *


3   P-3   4   GA   SP   *   118.32     31.29  *  118.32     31.29    *
3   P-3   5   GA   PP   *    79.08     41.79  *   79.08     41.79    *
3   P-3   5   GA   SP   *   118.32     31.29  *  118.32     31.29    *
3   P-3   6   GA   PP   *    61.73     34.87  *   66.32     33.63    *
3   P-3   6   GA   SP   *    92.36     27.79  *   99.23     24.12    *

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4   S-1   1   GA   PP   *    74.48     37.93  *   74.48     37.93    *
4   S-1   1   GA   SP   *    99.31     36.47  *   99.31     36.47    *
4   S-1   2   GA   PP   *    57.14     29.60  *   60.58     28.56    *
4   S-1   2   GA   SP   *    76.19     28.64  *   80.78     26.61    *
4   S-1   3   GA   PP   *    75.00     33.30  *   75.00     33.30    *
4   S-1   3   GA   SP   *   100.00     30.20  *  100.00     30.20    *
4   S-1   4   GA   PP   *    62.75     37.76  *   66.96     30.68    *
4   S-1   4   GA   SP   *    83.67     31.90  *   89.28     28.22    *
4   S-1   5   GA   PP   *    68.87     30.95  *   68.87     30.95    *
4   S-1   5   GA   SP   *    91.83     28.23  *   91.83     28.23    *
4   S-1   6   GA   PP   *    55.61     32.50  *   60.58     29.98    *
4   S-1   6   GA   SP   *    74.14     32.80  *   80.78     28.50    *

4   S-2   1   GA   PP   *    58.67     34.26  *   68.02     32.26    *
4   S-2   1   GA   SP   *    77.96     30.30  *   90.39     25.21    *
4   S-2   2   GA   PP   *    45.40     27.24  *   58.67     19.49    *
4   S-2   2   GA   SP   *    60.33     24.41  *   77.96     10.68    *
4   S-2   3   GA   PP   *    68.36     32.16  *   68.36     32.16    *
4   S-2   3   GA   SP   *    90.84     24.99  *   90.84     24.99    *
4   S-2   4   GA   PP   *    50.00     25.91  *   56.12     21.20    *
4   S-2   4   GA   SP   *    66.44     21.46  *   74.57     13.60    *
4   S-2   5   GA   PP   *    51.53     25.46  *   59.94     25.33    *
4   S-2   5   GA   SP   *    68.47     20.46  *   79.66     18.10    *
4   S-2   6   GA   PP   *    52.04     23.43  *   58.67     19.49    *
4   S-2   6   GA   SP   *    69.15     17.63  *   77.96     10.68    *

4   P-1   1   GA   PP   *    94.09     28.82  *   81.52     27.41    *
4   P-1   1   GA   SP   *    85.35     34.98  *   83.33     23.43    *
4   P-1   2   GA   PP   *    12.42     13.77  *                      *
4   P-1   2   GA   SP   *    11.26     13.66  *                      *
4   P-1   3   GA   PP   *   102.48     35.85  *   85.40     18.16    *
4   P-1   3   GA   SP   *    92.95     42.15  *   87.30     13.75    *
4   P-1   4   GA   PP   *   105.59     27.72  *                      *
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CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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PA = PLAIN MEMBRANE ON AGAR

SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L	H2O	M	S	T	RECOVERY - PERCENT OF STD			
A	SPL	F	T	S	ALL DATA		SELECTED DATA	
B	TYP	G	D	T	AVG	ERR	AVG	ERR
4	P-1	4	GA	SP	95.77	35.06		
4	P-1	5	GA	PP	65.52	16.91	65.52	16.91
4	P-1	5	GA	SP	59.43	21.49	66.98	13.59
4	P-1	6	GA	PP	66.14	22.05	66.14	22.05
4	P-1	6	GA	SP	60.00	26.21	67.61	18.81
4	P-2	1	GA	PP	126.54	68.27	134.04	62.56
4	P-2	1	GA	SP	40.28	25.65	50.00	20.77
4	P-2	2	GA	PP	139.82	50.95	134.46	47.65
4	P-2	2	GA	SP	44.50	20.55	50.15	15.20
4	P-2	3	GA	PP	114.15	31.77	109.78	29.45
4	P-2	3	GA	SP	36.33	13.65	40.95	8.88
4	P-2	4	GA	PP	100.00	29.48	100.00	25.86
4	P-2	4	GA	SP	31.83	12.48	37.30	7.73
4	P-2	5	GA	PP	96.46	35.27	103.54	31.23
4	P-2	5	GA	SP	30.70	14.22	38.62	9.67
4	P-2	6	GA	PP	112.38	39.22	114.89	32.39
4	P-2	6	GA	SP	35.77	15.97	42.85	9.89
4	P-3	1	GA	PP	144.73	44.96	137.50	34.39
4	P-3	1	GA	SP	46.47	20.39	52.38	14.67
4	P-3	2	GA	PP	111.40	60.65	129.16	50.00
4	P-3	2	GA	SP	35.77	24.06	49.20	20.52
4	P-3	3	GA	PP	142.10	42.11	135.00	31.84
4	P-3	3	GA	SP	45.63	19.37	51.42	13.67
4	P-3	4	GA	PP	86.84	25.52	91.66	11.13
4	P-3	4	GA	SP	27.88	11.77	34.92	5.29
4	P-3	5	GA	PP	121.05	30.26	115.00	21.79
4	P-3	5	GA	SP	38.87	14.70	43.80	9.61
4	P-3	6	GA	PP	122.80	58.72	131.25	36.44
4	P-3	6	GA	SP	39.43	23.91	50.00	15.38
4	P-4	1	GA	PP	214.03	58.72	203.33	43.48
4	P-4	1	GA	SP	154.43	110.42	112.61	47.27
4	P-4	2	GA	PP	142.10	55.25	135.00	44.32
4	P-4	2	GA	SP	102.53	85.05	74.76	39.94
4	P-4	3	GA	PP	317.54	100.36		
4	P-4	3	GA	SP	229.11	173.38		
4	P-4	4	GA	PP	228.07	56.72	216.66	40.77
4	P-4	4	GA	SP	164.55	113.45	120.00	47.29
4	P-4	5	GA	PP	238.59	72.50	216.66	51.83

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

TST/STD COLUMNS: GA = GRIDDED MEMBRANE ON AGAR
PA = PLAIN MEMBRANE ON AGAR
SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	*	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	 * *	 SELECTED DATA AVG	 ERR	* * *
4	P-4	5	GA	SP	*	172.15	128.17	*	120.00	53.42	*
4	P-4	6	GA	PP	*	289.47	75.17	*			*
4	P-4	6	GA	SP	*	208.86	146.28	*			*
5	S-1	1	GA	PP	*	763.15	205.21	*			*
5	S-1	1	GA	SP	*	100.11	40.85	*			*
5	S-1	2	GA	SP	*	76.00	23.14	*			*
5	S-1	3	GA	SP	*	131.46	49.65	*			*
5	S-1	4	GA	SP	*	66.51	46.00	*			*
5	S-1	5	GA	SP	*	94.76	18.08	*			*
5	S-1	6	GA	SP	*	122.90	40.18	*			*
5	S-2	1	GA	PP	*	71.31	24.42	*			*
5	S-2	2	GA	PP	*	54.28	20.42	*			*
5	S-2	3	GA	PP	*	58.16	23.01	*			*
5	S-2	4	GA	PP	*	35.15	27.27	*			*
5	S-2	5	GA	PP	*	103.05	32.00	*			*
5	S-2	6	GA	PP	*	81.48	41.48	*	73.68	14.10	*
5	P-1	1	GA	SP	*	156.41	37.34	*			*
5	P-1	2	GA	SP	*	159.85	113.77	*			*
5	P-1	3	GA	SP	*	211.12	115.59	*			*
5	P-1	4	GA	SP	*	191.78	84.91	*			*
5	P-1	5	GA	SP	*	259.50	155.43	*			*
5	P-1	6	GA	SP	*	297.80	100.47	*			*
5	P-2	1	GA	SP	*	129.28	70.02	*	116.77	53.49	*
5	P-2	2	GA	SP	*	158.47	29.71	*	158.47	29.71	*
5	P-2	3	GA	SP	*	196.61	78.66	*	161.86	38.40	*
5	P-2	4	GA	SP	*	133.24	28.25	*	133.24	28.25	*
5	P-2	5	GA	SP	*	186.77	69.31	*	172.19	53.43	*
5	P-2	6	GA	SP	*	157.89	56.53	*	147.36	43.50	*
5	P-3	1	GA	SP	*	98.13	33.16	*	98.13	33.16	*
5	P-3	2	GA	SP	*	104.62	53.62	*	92.59	40.43	*
5	P-3	3	GA	SP	*	136.71	47.19	*	119.16	29.45	*
5	P-3	4	GA	SP	*	107.32	22.63	*	107.32	22.63	*
5	P-3	5	GA	SP	*	108.02	32.60	*	108.02	32.60	*
5	P-3	6	GA	SP	*	152.77	64.61	*	136.88	30.07	*

CODES -

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Table I. Fecal Coliform Recovery Data (Cont'd)

L A B	H2O SPL TYP	M F G	S T D	T S T	*	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	*	 SELECTED DATA AVG	 ERR	*
5	P-4	1	GA	PP	*	78.49	11.91	*	78.49	11.91	*
5	P-4	2	GA	PP	*	80.89	22.11	*	80.89	22.11	*
5	P-4	3	GA	PP	*	108.89	29.63	*	108.89	29.63	*
5	P-4	4	GA	PP	*	87.09	23.68	*	87.09	23.68	*
5	P-4	5	GA	PP	*	102.51	24.85	*	102.51	24.85	*
5	P-4	6	GA	PP	*	100.74	17.06	*	100.74	17.06	*
6	S-2	1	GA	PP	*	97.68	38.12	*	97.68	38.12	*
6	S-2	1	GA	SP	*	106.28	43.17	*	106.28	43.17	*
6	S-2	2	GA	PP	*	80.30	40.20	*	97.34	24.57	*
6	S-2	2	GA	SP	*	53.80	39.63	*	69.79	39.00	*
6	S-2	3	GA	PP	*	128.87	59.05	*	120.90	48.96	*
6	S-2	3	GA	SP	*	95.00	46.72	*	95.00	46.72	*
6	S-2	4	GA	PP	*	78.12	46.85	*	96.35	20.21	*
6	S-2	4	GA	SP	*	75.75	52.36	*	93.43	28.15	*
6	S-2	5	GA	PP	*	98.03	30.92	*	98.03	30.92	*
6	S-2	5	GA	SP	*	82.57	26.92	*	82.87	26.92	*
6	S-2	6	GA	PP	*	126.31	53.47	*	126.31	53.47	*
6	S-2	6	GA	SP	*	124.85	94.07	*	111.48	74.62	*
6	P-1	1	GA	PP	*	81.84	83.86	*			*
6	P-1	1	GA	SP	*	92.11	133.94	*			*
6	P-1	2	GA	PP	*	4.89	11.65	*			*
6	P-1	2	GA	SP	*	4.37	12.00	*			*
6	P-1	3	GA	PP	*	121.44	91.54	*			*
6	P-1	3	GA	SP	*	111.71	126.30	*			*
6	P-1	4	GA	PP	*	58.22	22.87	*	58.22	22.87	*
6	P-1	4	GA	SP	*	66.02	34.48	*	66.02	34.48	*
6	P-1	5	GA	PP	*	65.33	39.53	*	66.93	11.86	*
6	P-1	5	GA	SP	*	89.13	93.34	*	76.71	30.55	*
6	P-1	6	GA	PP	*	131.74	64.40	*			*
6	P-1	6	GA	SP	*	251.29	197.60	*			*
7	S-2	1	GA	PP	*	86.07	10.30	*			*
7	S-2	2	GA	PP	*	5.82	1.86	*			*
7	S-2	3	GA	PP	*	92.65	10.30	*			*

CODES -

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SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	 SELECTED DATA AVG	 ERR
7	S-2	4	GA	PP	71.13	12.16	68.67	9.92
7	S-2	5	GA	PP	75.44	8.15	73.83	7.34
7	S-2	6	GA	PP	91.13	12.68		
7	P-1	1	GA	PP	94.63	8.49		
7	P-1	2	GA	PP	0.56	0.80		
7	P-1	3	GA	PP	90.11	12.00	84.74	5.24
7	P-1	4	GA	PP	77.96	9.22	77.96	9.22
7	P-1	5	GA	PP	88.13	11.97	81.92	7.06
7	P-1	6	GA	PP	85.87	13.69	76.97	5.76
7	P-2	1	GA	PP	94.51	12.10	94.51	12.10
7	P-2	2	GA	PP	85.65	10.21	85.65	10.21
7	P-2	4	GA	PP	72.15	10.70	72.15	10.70
7	P-2	5	GA	PP	75.94	11.92	75.94	11.92
7	P-2	6	GA	PP	77.21	17.13	77.21	17.13
7	P-3	1	GA	PP	89.14	19.08	86.34	16.77
7	P-3	2	GA	PP	1.31	2.30		
7	P-3	3	GA	PP	90.78	22.65	86.75	19.09
7	P-3	4	GA	PP	62.50	19.50	62.50	19.50
7	P-3	5	GA	PP	77.96	14.92	77.96	14.92
7	P-3	6	GA	PP	79.27	13.48	79.27	13.48
7	P-4	1	GA	PP	89.04	18.05	89.04	18.05
7	P-4	3	GA	PP	90.41	18.99	90.41	18.99
7	P-4	4	GA	PP	73.97	18.98	77.91	16.43
7	P-4	5	GA	PP	85.61	21.23	85.61	21.23
7	P-4	6	GA	PP	86.30	19.65	86.30	19.65
9	S-2	1	GA	PP	238.35	29.27		
9	S-2	1	GA	SP	84.26	27.45		
9	S-2	2	GA	PP	245.20	48.44		
9	S-2	2	GA	SP	86.68	34.72		
9	S-2	4	GA	PP	272.60	44.06		
9	S-2	4	GA	SP	96.36	35.14		
9	S-2	5	GA	PP	254.10	57.27		
9	S-2	5	GA	SP	89.83	38.46		
9	S-2	6	GA	PP	236.30	50.17		

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L	H2O	M	S	T	RECOVERY - PERCENT OF STD			
A	SPL	F	T	S	ALL DATA		SELECTED DATA	
B	TYP	G	D	T	AVG	ERR	AVG	ERR
9	S-2	6	GA	SP	83.53	34.69		
9	P-1	1	GA	PP	189.72	47.87	171.23	36.36
9	P-1	1	GA	SP	78.02	22.29	70.42	17.30
9	P-1	2	GA	PP	188.35	34.90	183.21	31.16
9	P-1	2	GA	SP	77.46	16.93	75.35	15.33
9	P-1	4	GA	PP	193.83	43.50	178.08	29.85
9	P-1	4	GA	SP	79.71	20.54	73.23	14.71
9	P-1	5	GA	PP	202.05	47.15	172.94	19.89
9	P-1	5	GA	SP	83.09	22.16	71.12	10.55
9	P-1	6	GA	PP	195.89	32.91	187.21	26.81
9	P-1	6	GA	SP	80.56	16.22	76.99	13.59
9	P-2	1	GA	PP	200.00	38.74	188.35	29.30
9	P-2	1	GA	SP	73.73	32.00	79.42	24.66
9	P-2	2	GA	PP	208.21	45.53	184.93	38.05
9	P-2	2	GA	SP	76.76	35.23	77.97	28.13
9	P-2	4	GA	PP	197.26	49.44	187.50	41.89
9	P-2	4	GA	SP	72.72	35.70	79.06	29.91
9	P-2	5	GA	PP	219.17	42.25	202.05	25.25
9	P-2	5	GA	SP	80.80	34.99	85.19	23.85
9	P-2	6	GA	PP	215.75	41.61	195.20	29.40
9	P-2	6	GA	SP	79.54	34.45	82.31	25.15
9	P-3	1	GA	PP	186.98	35.59	180.65	28.47
9	P-3	1	GA	SP	80.29	25.88	77.57	22.46
9	P-3	2	GA	PP	191.09	31.01	191.09	31.01
9	P-3	2	GA	SP	82.05	24.14	82.05	24.14
9	P-3	4	GA	PP	205.47	37.18	188.35	19.02
9	P-3	4	GA	SP	88.23	27.61	80.88	18.84
9	P-3	5	GA	PP	206.16	41.71	184.93	28.36
9	P-3	5	GA	SP	88.52	29.59	79.41	22.66
9	P-3	6	GA	PP	210.27	52.59	189.49	29.60
9	P-3	6	GA	SP	90.29	34.49	81.37	23.45
9	P-4	1	GA	PP	204.79	53.12	182.64	32.29
9	P-4	1	GA	SP	77.86	19.93	69.44	12.04
9	P-4	2	GA	PP	199.31	42.51	186.07	36.03
9	P-4	2	GA	SP	75.78	15.90	70.74	13.45
9	P-4	4	GA	PP	195.89	33.35	191.78	30.90
9	P-4	4	GA	SP	74.47	12.42	72.91	11.50

CODES -

H_2O SPL TYP COLUMN: P = POLLUTED WATER S = SEWAGE

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SP = SPREAD PLATE, PP = POUR PLATE

Table I. Fecal Coliform Recovery Data (Cont'd.)

L	H2O	M	S	T	RECOVERY - PERCENT OF STD			
A	SPL	F	T	S	ALL DATA		SELECTED DATA	
B	TYP	G	D	T	AVG	ERR	AVG	ERR
9	P-4	5	GA	PP	191.78	45.45	183.21	38.96
9	P-4	5	GA	SP	72.91	17.03	69.66	14.57
9	P-4	6	GA	PP	180.13	37.04	172.94	28.82
9	P-4	6	GA	SP	68.48	13.85	65.75	10.73
10	S-2	1	GA	PP	121.63	44.90	121.63	44.90
10	S-2	1	GA	SP	54.16	15.68	54.16	15.68
10	S-2	2	GA	PP	131.49	53.12	118.09	25.27
10	S-2	3	GA	PP	130.40	41.07	130.40	41.07
10	S-2	4	GA	PP	111.69	34.16	111.69	34.16
10	S-2	5	GA	PP	103.50	37.12	103.50	37.12
10	S-2	6	GA	PP	143.09	36.19	143.09	36.19
10	P-1	1	GA	PP	152.55	43.56	152.55	43.56
10	P-1	2	GA	PP	107.82	68.74		
10	P-1	3	GA	PP	128.94	42.12		
10	P-1	4	GA	PP	135.00	55.00		
10	P-1	5	GA	PP	152.72	50.62	133.63	26.07
10	P-1	6	GA	PP	156.57	39.49		

CODES -

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Table I. Fecal Coliform Recovery Data (Cont'd.)

L A B	H2O SPL TYP	M F G	S T D	T S T	RECOVERY - PERCENT OF STD ALL DATA AVG	 ERR	 SELECTED DATA AVG	 ERR
9	S-2	6	GA	SP	83.53	34.69		
9	P-1	1	GA	PP	189.72	47.87	171.23	36.36
9	P-1	1	GA	SP	78.02	22.29	70.42	17.30
9	P-1	2	GA	PP	188.35	34.90	183.21	31.16
9	P-1	2	GA	SP	77.46	16.93	75.35	15.33
9	P-1	4	GA	PP	193.83	43.50	178.08	29.85
9	P-1	4	GA	SP	79.71	20.54	73.23	14.71
9	P-1	5	GA	PP	202.05	47.15	172.94	19.89
9	P-1	5	GA	SP	83.09	22.16	71.12	10.55
9	P-1	6	GA	PP	195.89	32.91	187.21	26.81
9	P-1	6	GA	SP	80.56	16.22	76.99	13.59
9	P-2	1	GA	PP	200.00	38.74	188.35	29.30
9	P-2	1	GA	SP	73.73	32.00	79.42	24.66
9	P-2	2	GA	PP	206.21	45.53	184.93	38.05
9	P-2	2	GA	SP	76.76	35.23	77.97	28.13
9	P-2	4	GA	PP	197.26	49.44	187.50	41.89
9	P-2	4	GA	SP	72.72	35.70	79.06	29.91
9	P-2	5	GA	PP	219.17	42.25	202.05	25.25
9	P-2	5	GA	SP	80.80	34.99	85.19	23.85
9	P-2	6	GA	PP	215.75	41.61	195.20	29.40
9	P-2	6	GA	SP	79.54	34.45	82.31	25.15
9	P-3	1	GA	PP	186.98	35.59	180.65	28.47
9	P-3	1	GA	SP	80.29	25.88	77.57	22.46
9	P-3	2	GA	PP	191.09	31.01	191.09	31.01
9	P-3	2	GA	SP	82.05	24.14	82.05	24.14
9	P-3	4	GA	PP	205.47	37.18	188.35	19.02
9	P-3	4	GA	SP	88.23	27.61	80.88	18.84
9	P-3	5	GA	PP	206.16	41.71	184.93	28.36
9	P-3	5	GA	SP	88.52	29.59	79.41	22.66
9	P-3	6	GA	PP	210.27	52.59	189.49	29.60
9	P-3	6	GA	SP	90.29	34.49	81.37	23.45
9	P-4	1	GA	PP	204.79	53.12	182.64	32.29
9	P-4	1	GA	SP	77.86	19.93	69.44	12.04
9	P-4	2	GA	PP	199.31	42.51	186.07	36.03
9	P-4	2	GA	SP	75.78	15.90	70.74	13.45
9	P-4	4	GA	PP	195.89	33.35	191.78	30.90
9	P-4	4	GA	SP	74.47	12.42	72.91	11.50

CODES -

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Table 2 ANOVA TABLE – SPREAD PLATE STANDARD

Source	Sum of Squares	DF	Mean Squares	F	
Filters (F)	10257.6269	5	2051.5253	2.51	Significant - 97.5%
Samples (S)	22436.6289	4	5609.1572	6.87	*
FS	9214.7500	20	460.7375	0.56	*
Labs (L)	39769.3829	2	19884.6914	24.37	Significant - 99+%
FL	7386.6250	10	738.6625	0.90	Not Significant
SL	74822.8908	8	9352.8613	11.46	*
Residual	32624.6289	40	815.6157		
Total	196512.5316	89			

* Meaningless - since they refer to differences attributed to samples which we know are different.

Table 3 ANOVA TABLE – POUR PLATE STANDARD

Source	Sum of Squares	DF	Mean Squares	F	
Filters (F)	14464.0000	5	2892.8002	3.12	Significant - 97.5%
Samples (S)	21314.5000	4	5328.6250	5.74	*
FS	10630.3769	20	531.5188	0.57	*
Labs (L)	47449.2579	2	23724.6289	25.58	Significant - 99+%
FL	8669.1269	10	866.9125	0.93	Not Significant
SL	104435.5158	8	13054.4394	14.08	*
Residual	37086.0000	40	927.1500		
Total	244048.7816	89			

* Meaningless - since they refer to differences attributed to samples which we know are different.

Table 4 COMPONENTS OF VARIANCE ANALYSIS – SPREAD PLATE STANDARD

$$\sigma^2 \text{ Residual} = \frac{7386.625 + 40011.245}{50} = 800$$

$$\sigma^2 \text{ Lab} = \frac{19885 - \sigma^2 \text{ Residual}}{30} = 636$$

$$\sigma^2 \text{ Filters} = \frac{2052 - \sigma^2 \text{ Residual}}{15} = 83$$

σ^2 For a single determination on any filter in any laboratory is:

$$\sigma^2 = \sigma^2 \text{ Residual} + \sigma^2 \text{ Lab} + \sigma^2 \text{ Filter} =$$

$$800 + 636 + 83 = 1519$$

$\sigma = \pm 39$ $2\sigma = \pm 78$

GRAND MEAN = 81.5

Table 5 COMPONENTS OF VARIANCE ANALYSIS – POUR PLATE STANDARD

$$\sigma^2 \text{ Residual} = \frac{8669. + 37086}{50} = 915$$

$$\sigma^2 \text{ Lab} = \frac{23725 - \sigma^2 \text{ Residual}}{30} = 760$$

$$\sigma^2 \text{ Filters} = \frac{2893 - \sigma^2 \text{ Residual}}{15} = 132$$

σ^2 for a single determination on any filter in any laboratory is:

$$\sigma^2 = \sigma^2 \text{ Residual} + \sigma^2 \text{ Lab} + \sigma^2 \text{ Filter} =$$

$$915 + 760 + 132 = 1807$$

$\sigma = \pm 42.5$ $2\sigma = \pm 85$

GRAND MEAN = 95.5

CRITIQUE ON ASTM TEST FOR RECOVERY OF FECAL COLIFORMS AND PROPOSAL FOR MODIFIED METHOD

Norman H. Goddard
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ABSTRACT

As both a manufacturer of membrane filters and as a participating laboratory, in the ASTM Collaborative Study on MF's, Sartorius (Goddard), made the following suggestions for change in the study plan:

1. Conduct the study with a flora more favorable than polluted waters in sewage. For example, use a potable water spiked with a standard pure culture.
2. Standardize sample mixing conditions.
3. Require separate counts of coliforms and non-coliforms.
4. Randomize sample positions in the incubation.
5. Verify accuracy of thermometer and incubator settings.

The requirements of a routine method for the testing for the recovery of fecal coliforms include the following:

Agreement must be obtainable with a comparison method such as the pour plate method.

The results must be reproducible irrespective of the location of the testing laboratory or of the person performing the test.

Evaluation must be clear, without the possibility of subjective errors, so that the culture medium must not only be selective but also allow simple recognition of the resulting colonies.

The method itself must be simple and practical, capable of use in any testing laboratory.

As the leading European manufacturer of membrane filters, we welcome this attempt by the American Society for Testing and Materials and the United States Environmental Protection Agency to standardize a method for testing the suitability of membrane filters for fecal coliform determinations, and for comparing different brands of membrane filters. We were therefore very pleased to be invited to participate actively in this work and to carry out the round robin comparison tests on various filters. Such a filter comparison requires that all influences which can affect the result, excepting the filters, be removed. Unfortunately, we believe that this was not the case with the procedure used, and that modifications are required for meaningful results to be obtained.

One point of interest which arose from our tests was, however, that there appeared to be some correlation between the flow rate through a filter under standard conditions and the efficiency of recovery of that filter. The faster the flow rate, the higher the efficiency.

We heard yesterday that higher recoveries can be obtained by increasing the average surface opening diameter of the filter to an optimum size. Now the surface opening diameter is directly related to the pore size. The former is measured by electron microscopy, the latter is characterized by the mercury intrusion method, by retention characteristics or by flow rate measurements. In general, the faster the flow rate, the larger the pore size, and the larger the average surface opening diameter.

It was also stated yesterday that a reason for the higher recovery with a larger pore size filter may be the better provision of nutrient to a microorganism sitting in a surface pocket compared to one perched on the top of the filter and exposed to different evaporation effects.

The structure of a membrane filter is uniform, the pore size being the same throughout the width of the filter. Another possibility is therefore that the use of a larger pore size filter simply results in an easier passage of the nutrient from the bottom to the top surface of the filter, thus favoring growth.

Our experiments in this direction are not yet completed, but it could be that an optimum surface opening diameter, or pore size, for the recovery of fecal coliforms can be decided upon, and that comparisons between competitive filters need then be made only on the basis of the measurement of physical parameters: the sizes, and the reproducibility of the sizes, of the surface openings or pores on each side of the filter, and the flow rate through the filter under defined conditions.

This is purely conjecture at present, and I will therefore return to the test under discussion.

We are not happy with the samples suggested, polluted water and raw sewage, as a number of E. coli and coliform types can occur which affect the reported recovery because of subjective evaluation. Different investigators may not agree as to which colonies are fecal coliforms.

Additionally, such samples can contain high concentrations of sub-lethally injured microorganisms, and there is a large variation in the samples taken at the different locations of the investigating laboratories.

The culture medium, M-FC agar, is apparently not sufficiently selective. Not only coliform colonies but also numerous other colonies are obtained, which may or may not work as antagonists. either by affecting the growth of the bacteria we are searching for, or by changing the pH of the surrounding nutrient by alkali formation and so affecting blue color formation.

We filtered lake water through a filter and cultivated as usual. We then selected four of the blue colored colonies and three different non-blue colonies and subcultured them.

We streaked the blue colony culture on each of three different M-FC agar plates and streaked the culture of the non-blue colonies vertically to the blue colonies, one on each of the three plates.

On incubating, differing effects of the non-blue on the blue colonies could be seen. This shows that according to the type and the amount of alkali producing bacteria which are present, the typical blue color of the coliforms may be weakened or even completely prevented from occuring.

Fewer non-coliforms appear to grow on Endo media than on M-FC and strong alkali producers appear to have less effect.

Returning to the present procedure, the length of incubation using M-FC agar appears to be insufficient, as we have often noted blue color developing after completion of the standard incubation time. The incubation temperature of 44.5 C. is difficult to maintain in routine use.

With regard to the reference method, using samples containing high concentrations of antagonists, their effect depends on the contact area with the nutrient. The relationship of sample volume to contact area should be the same with the filter and the reference method, i.e. smear plates should be of the same diameter as the filter diameter used. Pour plates of the same diameter have a far larger contact area, and conditions within the culture medium may affect different antagonists in different ways.

The sample suggested, polluted waters, contain bacterial agglomerates. Shaking will dispense them to an extent, depending on the amount and method of shaking. Some aggregate break-up occurs in the pour plate method on mixing the sample with the warm viscous agar.

We would therefore suggest that a sample be used which contains favorable bacteria flora for this test. We recommend one in which the numerous types of protein decomposing, alkali forming bacteria occurring in raw sewage do not occur, so that the difference in contact area between test and reference method is not important.

Such a sample could be potable water with added pure culture.

The conditions of shaking the sample are less important with potable water than with polluted water but should nevertheless be standardized.

M-FC agar would be suitable as culture medium for this sample, whereas with a sample containing a bacteria spectrum approaching that of raw sewage a more specific culture medium is required. Regardless of which culture medium is used, the total number of non-coliform colonies occuring should be stated in addition to the coliform count.

Again, using such a sample, the incubation time of 22-24 hours would be sufficient and difficulty in evaluation would not occur.

Streaked spread plates of the same diameter as the filter would be optimal, but not practical because of the small sample volume which can be spread. Pour plates in 60 mm. Petri dishes should therefore be used with a good relationship between sample volume and contact area.

Randomization of sample positions during incubation is necessary, as is a check on the accuracy of the thermometer used.

QUESTIONS AND ANSWERS

Geldreich: Was rosolic acid added to the medium which you used?

Goddard: (I don't really know.) I must apologize, I am not a microbiologist and if I were timid, I would not have read this technical paper, but we did want to comment on comparisons of membrane filters.

Geldreich: I would like to know if rosolic acid was added, to cut down the background of other organisms?

Bordner: The method carried out is exactly as in the ASTM draft procedure given us and the draft procedure did include the addition of rosolic acid. Any other questions related to this paper?

Brezenski: One of the criteria established in the comparison of the replicate membrane filters was an 85% recovery. Why was this number chosen - what is the basis for it? I just want to know what the reference is, that's all.

Bordner: This was just a goal that was set arbitrarily by the subcommittee. I think the percentage was from the Department of Defense specifications for membrane filters. That percentage was also quoted for previous recovery tests that were identified in the DOD specs that we discussed yesterday. Any other questions?

Litsky: I cannot leave this room without asking the question I asked yesterday morning, and that is the problem of the extractables. We have perfectly good procedures on extractables. If the specifications require changing, let's try to get a uniform product. If the definition needs changing, let's change it. But its rather ironic that three months ago I sat in an EPA committee concerning disinfectants and detergents, and they stuck to the letter of the law! Any deviation from the specifications caused a riot. If the specs written ten years ago do not apply now, let's change them.

Sims: During the last two days we have seen a dilemma develop for the manufacturer. We are responsive to you, the customer, and we can provide you with whatever you want. The representative from Millipore pointed out this morning that if you create this surface - what they are calling greater surface pore size - you create a different atmosphere. It can be provided. Also, cells in the surface cavity will have a tendency to run and colonies won't look the same. In purchasing membranes you are going to have to be more particular about what results you require and not as particular about cell appearance.

The specs were written ten years ago and our membrane was developed to meet these specs, whether correct or not, it is the law. If you are selling to the US Army, they buy by those specs. You live with extractables. Everybody does the same thing. If somebody adds more extractables and they can justify it and give you a product, fine. If you want zero extractables, the membrane can be washed. It will be brittle and it might have usage effects that would really turn you off. You, the customer, are deciding what you want to buy. The manufacturer will meet your demands. What we want from this symposium is to find out what your demands are.

Right now, from the attitudes I have seen, you want specific microbiological properties. You will accept, perhaps, not as even a gridline, not as dark a print. You will take perhaps a little more brittle product or a product packaged in a different way. Maybe you want to autoclave the membrane and it wrinkles, maybe it doesn't. I think what we have been discussing is a two-way street. I am looking forward to getting the results of the committee meeting over the next few days and as manufacturers we will provide what you want.

The thing that did interest me about this surface phenomenon is that membranes can be manufactured to create a very tight system, a uniform pore throughout where bacteria sit right on the top, fluid has to nuture the cell from both sides and the cell grows into a little round colony. If you have a sponge-like network the cell is down in it, the fluid surrounds it almost on four sides, but always on three, the colony growth might spread out differently, depending on the structure. If you are affected drastically by colony appearance and how easy it is for your technician to count, you might get lower counts. It's something, I think, that is very vital. The manufacturer can provide you with either structure - its no problem.

The only other question I have for the representative from Millipore concerns the 0.7 μm filter. Will **Serratia marcescens** still be the species used to standardize filters? This is what the specs now read and as a manufacturer I live by these specs. Are we going to vary the way we control retention?

Bordner: We hope to provide some of these answers in the ASTM committee meeting.

SUMMARY OF SYMPOSIUM

John A. Winter, Chief, Quality Assurance Branch
Environmental Monitoring and Support Laboratory, Cincinnati, EPA
and
Francis T. Brezenski, Chief, Technical Support Branch
Edison Water Quality Laboratory, Edison, EPA

SUMMARY AND DISCUSSION

Bordner (Moderator) — This morning the agenda is shorter than yesterday. We plan to end the Symposium by noon. It will be followed this afternoon by a meeting of ASTM subcommittee D19.08.04.02. You are welcome to stay this afternoon and participate. You are also encouraged to join the subcommittee as permanent members.

The last item on the program this morning is a summarization of the papers in the Symposium. It will be given by Fran Brezenski and John Winter, both microbiologists in EPA.

The papers are placed into four categories, however, the audience should be aware that there is a considerable overlap of subject matter between categories.

I. General papers.

II. Stressed/injured cells and observed effects on MF recoveries.

III. Comparisons between MF lots and brands.

IV. Solutions to apparent low recoveries.

I. General Papers

1) Bordner - The Membrane Filter Dilemma

Bordner described the general preference in water analyses for MF techniques based on simplicity and speed. Increasingly, however, the literature has reported low or variable MF results, particularly for fecal coliform bacteria and has attributed these results to brand or lot differences in MFs, stressing of bacteria in natural waters, inhibitive incubation at 44.5 C, chlorination, and other test factors.

ASTM Committee D-19 and the U.S. EPA sponsored this seminar to: disseminate known information on the problem, identify the better possible solutions, establish the standard test procedures needed for validly comparing media, membrane filters, and other test factors, and select the best solution using these standard tests.

2) Geldreich - Performance Variability of Membrane Filter Procedures

The variable results reported from membrane filter procedures are ascribed not only to differences in membrane filter materials and methods of MF sterilization but also to inconsistencies in absorbent pads, commercially-prepared media, and the knowledge and experience of the technician. The need for cooperation between manufacturers and users and the need for improved quality control programs by both were emphasized.

3) Powers - Quality Control of Media

Powers reviewed the manufacturing techniques and the quality control techniques practiced by the manufacturer. He suggested that the manufacturer loses control over product quality once it leaves his plant. He then described the abuses in laboratory test conditions which influence microbial recoveries, growth and colonial characteristics and stated that most media problems result from mishandling of media in the laboratory. This attitude moves final QC responsibility from the manufacturer to the user.

4) Sladek - Statistical Interpretation of MF Counts

Sladek cited the importance of statistical design in any comparison of microbial recoveries and reviewed the main factors to be measured in such studies.

He used examples of actual studies to describe measures of precision and to demonstrate the importance of using large numbers of replicates. He cited the problems of stabilizing samples for true replication over time. Sladek emphasized that the theoretical minimum deviation inherent in the method must be established to compare with subsequent experimental deviations. He stressed that valid studies can only be made by removal or control through randomization of unwanted variables which include sampling, types of samples, procedures, materials and media.

Finally, Sladek recommended the use of a control which is independent of the test methods, i.e., non-MF control for comparing MF tests.

II. Injury Papers/Effect Papers

1) Hoadley - Effects of Injury on Recovery of Indicators on Membrane Filters

Hoadley studied the recovery of **E. coli** and **S. faecalis** as control cultures and as cultures stressed by exposure to stream conditions and to chlorination. He used spread plate and membrane filter tests on selective and non-selective media. Control cultures of **S. faecalis** showed low recovery on selective media while **E. coli** controls recovered well.

After stressing, **E. coli** yielded low recovery on MFs, while **S. faecalis** showed good recovery. **E. coli** counts on MFs were reduced with increased stress.

Hoadley concludes **S. faecalis** may be a better indicator than **E. coli** because it does not show low recovery after stress. Further, he recommends that any studies for improved methodology should include evaluation of stressed cell populations.

2) Hufham - Effects of Temperature on Recovery of Fecal Coliform

Hufham cites extreme effects of temperature on recovery of fecal coliforms, suggesting that these high temperature effects (44.5 C) can overlap effects of membrane differences. Temperature may have been a major factor in earlier papers citing severe differences in membranes.

For future recovery studies, Hufham proposes the use of a standard **E. coli** culture which has not been selected from MF cultures at 44.5 C. He proposes that a standard plate count of cells grown at 35 C is necessary to avoid temperature-related losses and to establish the reference cell numbers for MFs or other comparisons.

III. Comparison Papers

1) Brodsky - A Comparison of Membrane Filters and Media Used to Recover Coliforms from Water

Two to seven laboratories analyzed water samples and cultures for coliform bacteria using membrane filters from Johns-Manville of Canada, Millipore Corporation and the Sartorius Company.

Cultures were both routine water samples and total and fecal coliform mixed cultures obtained by passage through MacConkey broth and EC broth. Analyses were performed in parallel using LES Endo Agar and M-Endo agar.

Although the Johns-Manville and Millipore filters showed higher recoveries than Sartorius in Phase I of the study, Phase II results showed the three filters to be equal. Recoveries on LES Endo agar and M-Endo agar were similar. The authors reported that their results varied with the test conditions used. They concluded that test design and quality control must be carefully selected and standardized before the results are meaningful.

2) Stuart - Comparison of MFs in Recovery of Naturally-Injured Coliforms

In a series of studies, Schillinger **et al.** exposed pure cultures of **E. coli** using MF chambers, **in situ** in a stream. Cells were recovered, enriched and tested by the M-FC procedure, using Gelman, Millipore and Nuclepore filters. Results to date show no significant difference in recovery of **E. coli** on Gelman or Millipore filters. Nuclepore filters gave lower recoveries than the other brands. It was concluded the Nuclepore brand should not be used for culture work.

3) Harris - Efficiency of Coliform Recovery Using Two Brands of MFs

Harris and Bailey compared three lots each of Gelman and Millipore filters in the testing of aerobic lagoon and sewage samples for total and fecal coliform bacteria. Gelman filters were steam-autoclaved and Millipore filters were sterilized with ethylene oxide. Total coliform counts showed no significant difference with lot or brand. However, twice as many fecal coliforms were recovered on Gelman filters as on Millipore filters. Colonies on Gelman filters were smaller than on Millipore. Millipore filters were blue and Gelman filters beige, suggesting a pH effect on the dyes.

This work confirms Presswood and Brown's work. In later work by Harris and Bailey, experimental membrane filters from Millipore showed recoveries comparable to Gelman. The authors urge more quality control for uniformity between batches and brands.

4) Dufour - Comparison of MF Brands for the Recovery of the Coliform Group

Dufour and Cabelli studied the recoveries of pure cultures of **E. coli, K. Pneumoniae** and fecal and total coliforms from natural samples, using membrane filters produced by Gelman, Millipore, S&S, Sartorius and Nuclepore.

They found that strain differences in the organisms and differences between lots of membranes confused the recovery comparisons between brands. They noted that the precisions were consistent with brands when using pure cultures.

The accuracy of recoveries was the same whether pure cultures or natural samples were used. Because precision decreased with natural samples, studies done with these samples should use a larger number of replicates than for pure cultures. Acceptable total coliform counts were obtained with all filters except Nuclepore.

5) Glantz - Comparison of Millipore and Gelman Filters, Culture Media, Incubators and Escherichia coli strains

Pure culture isolates of **E. coli** were tested for recovery on Trypticase soy agar (TSA) pour plates, violet red bile agar (VRB) spread plates and M-FC broth membrane filtrations, using different brands and lots of membrane filters. Recoveries were compared at incubation temperatures of 35 C, 43 C and 44.5 C.

Although some cell counts were reduced at 44.5 C, counts varied most significantly between **E. coli** strains and between different MFs. M-FC broth gave lower recoveries than VRB. Standard strains of **E. coli** are recommended for future evaluations.

6) Davis - The ASTM Proposed Membrane Filter Test Procedure for the Recovery of Fecal Coliforms

Jackson and Davis described the preliminary test procedure developed by the ASTM subcommittee 019.08.04.02 for recovery of fecal coliforms by membrane filters and detailed the round robin test performed by ten laboratories using the procedure. They summarized the statistical analysis of the results and concluded that the preliminary procedure did detect differences in filters but was unsatisfactory because it did not separate the effects of individual laboratories and techniques from the natural sample differences and other procedure variables.

7) Goddard - Critique on ASTM Test for Recovery of Fecal Coliforms and Proposal for Modified Method

Goddard feels that the necessary standardization and control of variables was not attained in this first ASTM test effort.

He discussed briefly membrane characteristics and suggested that the better recovery reported with large surface pores could be due to the cradling effect or simply be due to easier passage of nutrients. He suggested that an optimal pore size for fecal coliforms be established as a standard and comparisions of filters then made based on other physical characteristics of the filter.

Goddard critiqued the ASTM procedure with the following suggestions for improvement: Use a standardized potable water sample to obtain a more uniform and favorable flora for the test, report noncoliform and coliform counts, use spread plates of same size as filters for fairer establishment of reference count, place samples randomly in incubator and verify accuracy of thermometers.

IV. Solutions

1) McFeters - Recovery Characteristics of Bacteria Injured in Natural Aquatic Environment

Bissonnette, et al. continued the MF chamber studies described by Schillinger, et al.. They placed raw sewage, E. coli and S. faecalis cultures in the chambers in situ in stream, and sampled the chambers daily over time. They plotted recoveries in Trypticase soy yeast extract agar (TYSA) and desoxycholate lactose agar (DLA) and calculated the number of bacterial cells injured (unable to grow).

Further studies added MPN lactose broth, MPN BGB broth, M-Endo MF and M-FC membrane filter media. Comparative recoveries were:

MPN - TSY = MPN - LB > MPN - BGB > DLA > M-Endo-MF > M-FC-MF

The numbers of survivors increased with increased time of exposure. Membrane filter media were less efficient than other procedures in recovering injured bacteria. Exposure of uninjured cells to TSY broth repaired the cells so that they could reproduce. A two-hour enrichment on TSY agar was suggested before enumeration of indicator bacteria on a selective medium.

2) Geldreich - An Improved MF Method for FC Analyses

Rose et al. described the development and testing by three laboratories of a two-layer agar method. The procedure used an overlay of lactose broth agar on M-FC agar and two-hour incubation at 35 C prior to the 44.5 C incubation for 22-24 hours. Limited work with raw and chlorinated wastewaters, and results from reservoir, stream and marine samples showed improved recoveries in 59 of 61 samples and a median improved recovery ratio of 1.9. More work must be done to verify these results.

3) Grasso - Measurement of Fecal Coliform in Estuarine Water

Stevens, Grasso and Delaney cited attempts to improve recoveries of fecal coliform from seawater using Millipore filters in two-step procedures. The first series using minimal media at 25 C failed to yield consistent recoveries.

The second approach utilized a two-step, two-day procedure of incubation at 25 C for 18 hours on a minimal LES medium then transfer to M-FC for incubation at 44.5 C for 24 hours. Grasso reported an average increase of 2.9 in the recovery of fecal coliforms as compared with EC counts. Ninety-three percent of the picks of fecal coliform colonies did verify.

Later work suggested that batch and brand differences in membrane filters may have influenced the study data which was generated using Millipore filters only. Comparison of Millipore and Gelman filters showed a high level of dissolved solids in the Gelman filters. Examination of water extracts from the filters showed a low pH and significant mg/liter levels of ammonia-nitrogen and orthophosphate in the Gelman filters. Gelman filters showed higher recoveries than Millipore filters on regular M-FC medium but lower recoveries with the new LES procedure. Further tests are being conducted.

4) Lin - Evaluation of Method for Detecting Coliforms and Fecal Streptococci in Chlorinated Secondary Sewage Effluents

In a massive series of tests, Lin studied total coliform, fecal coliform, and fecal streptococci recoveries from unchlorinated waters and wastewaters and chlorinated effluents, using membrane filters and MPN techniques.

MF recoveries with chlorinated secondary effluents were significantly lower for total coliform, fecal coliform and fecal streptococci than the counts by the standard MPN. M-FC counts were lower than EC recoveries. No fecal coliform enrichments were reported.

Low recoveries of total coliforms using a single-step procedure were improved 1.5 times by use of the LES two-step procedure and were comparable to the MPN values for these effluents. Equal recoveries of total coliforms were made with the LES two-step MF procedure and the standard MPN confirmed test when testing chlorinated secondary effluents.

Use of a bile broth enrichment for two and three days increased fecal streptococci counts to a level comparable to MPN values.

5) Sladek - Optimim Membrane Structures

Sladek studied test factors affecting recovery of fecal coliforms in MF tests and concluded that the most critical factor in recovery was the structure of the membrane filter. Studies on maximum recovery of fecal colifrom bacteria showed that there was an apparent optimum top surface opening of 2.4 μm accompanied by a 0.7 μm internal pore size. This surface opening was reported as critical for maximum recovery of fecal coliform organisms because the cells are exposed to a high evaporation effect at 44.5 C. Evaporation produces hypertonic solution areas unless the cells are cradled in surface openings. Millipore feels this theory explains the low M-FC recoveries on 0.45 μm membranes. The better recoveries on the new 2.4 μm surface pore, 0.7 μm internal pore membranes confirm this. Data are limited and must be substantiated.

GENERAL COMMENTS

Winter: Let's forget about any more comparisons of MF's. There have been enough such studies. If we do any more work we had better look at the solutions suggested in this Symposium.

Almost all papers given here were focussing on some variable and ignoring another variable of lot, batch, medium, incubation temperature, time and so forth. A number of solutions were proposed and these proposals need more supportive data, but only after factors which affect the MF test are controlled or randomized as described by Karl Sladek, so that we can get a valid comparison of numbers.

We would urge everyone to participate in collaborative studies performed with ASTM, EPA and other testing groups. No proposed method should be accepted until it has had a satisfactory collaborative study. Failure to do so was how mistakes were made in the past. If methods get into books, there is a great deal of difficulty getting them out.

At this stage we shouldn't try for an instant solution. We have a serious problem of test recoveries made more serious because of compliance monitoring regulations promulgated by EPA and implemented by the States. Specific industrial and municipal dischargers are required to get NPDES permits and prove that they are not exceeding the limits for fecal coliforms set in their permit. The fecal coliform test that must be used at this time for chlorinated wastes is the MPN. The MPN is the only technique we know of which seems to withstand the effects of chlorination and give us the maximum recoveries from chlorinated effluents.

Editor's Note: Since the time of the Symposium in January 20-21, 1975 EPA has reviewed public response to the proposal that only the MPN be used to analyze effluents for fecal coliforms in the presence of chlorine. The current proposal is that both the MPN and MF may be used but the method used must be identified. The MPN is the method of choice if controversy is anticipated because the MF has been reported to yield low and erratic results from chlorinated effluents.

Winter: The users need the cooperation of the MF manufacturers in solving the problem of recoveries on MF's. If they come out with a new formulation of membranes such as the one spoken of yesterday, the HC membrane filter from Millipore, I hope that we don't find out that it has phosphate or some other stimulating factor in it. We don't want more surprises about membrane filters. We were told that membrane filters were inert. The specs have always said they shall not enhance or inhibit growth. What should we do now? If user and supplier exchange information and work together they can find hopefully a solution to the MF recovery problems.

Brezenski: Bob Bordner started the Symposium with a question: why are we here? I think the answer was to cope with the problems of the lack of recovery by the membrane filter systems, and the number of conflicting reports in the literature. After reviewing the papers and tapes and listening to the discussion yesteraday we concluded those two objectives were met. So from that point of view, the meeting was successful, but the ASTM Committee on MF's should continue its work. I would like to make a suggestion to the manufacturers. One company contributed two papers to the Symposium. There are other companies manufacturing MF's. I would like to see a greater research contribution by the companies themselves. I am sure that most of them develop data in the process of controlling the membrane manufacturing process. This data can contribute to the knowledge about the physical and chemical characteristics of the membranes.

A number of papers described the phenomenon of the stressing of cells. There were a number of terms used yesterday: stressed cells, attentuated cells, resuscitated cells, damaged cells, debilitated cells, unresuscitated cells. All of these terms have a somewhat different connotation. I would urge some group, government, academic or private, to investigate what these debilitated cells are physiologically. Once we have this information, the medium and the whole testing procedure can be modified to recover these cells. Right now we see a lag time in growth and say that the cells were debilitated or they were stressed. In what way? This is a major problem that was not discussed in the Symposium. Yesterday, Ed Geldreich mentioned the tremendously large number of MF tests run in the US each year. It brings to mind that if we have defective MFs or problems of recovery, we have had many defective numbers produced and used over these past years.

I would like to close by saying that it was an enjoyable experience. I have been in a lot of other meetings and this is the first time I have seen so many worthwhile contributions and such active participation in a one-day session. I think we are well on our way to solving this problem. Thank you.

FINAL DISCUSSION

Cotton: I would like to commend the reviewers for putting the program of yesterday in such great perspective. I would like to speak briefly on behalf of membrane manufacturers. I was with Millipore in the beginning when the company first took the initial contract and developed the membrane. In fact, I was responsible for part of the development and for the initial work on the coliform test done by Millipore. One point which was left out was that although MF's are a big business for other purposes, the commercial development of the membrane filter stemmed from the coliform test. It was felt that the coliform test provided a significant commercial use for the membrane filter. This is where it all started. Although the use of the membrane filter in sanitary bacteriology is not the major commercial application, in spite of the millions of tests that are being run, it is a significant one and should be of great concern to the membrane manufacturers.

Mr. Geldreich made a point on filter specifications and quality control that

I think needs clarification. I know that our Company and other membrane manufacturers are aware of the need for extreme quality control techniques in their own laboratories. The problem is that there are many aspects of the filter that need to be controlled. Sometimes when you find a little piece of paper, as we saw yesterday, or when a box of filters comes out of production without the filters and only the separator pads, you can get a good laugh, but this represents a very small percentage of the total products produced. It is only one of about 14 different aspects which need careful control. All the manufacturers have brought some fine control conditions to the filter manufacturing process.

One point brought out was extractables in filters. I don't think this was completely clarified yesterday. One manufacturer's representative pointed out that glycerol was put into the filter to plasticize it. This is not correct. Glycerol is used in pore formation. It is a means to control the pore size of the filter, and after manufacture it is removed. The degree of removal will have an effect on the elasticity of the filter and for certain applications it is not important to remove all of the glycerol or even most of it. For some applications however, it is very important, especially in gravimetric analysis. In the case of the coliform test, if too much glycerol remains in the filter, false positives will result because glycerol is broken down to aldehydes which react with the fuchsin sulphite system and produce sheen. So, it is very important to limit the amount of glycerol that remains in the filter. This is one of the reasons for the tight government specifications. Originally the specifications on total extractables in the membrane were set at 2.5% Recently, the extractable condition on specifications in the government specs was dropped, and we are looking into this. We think it should be re-instituted primarily because of the false positives in the coliform test. Also, in the gravmetric analysis, which is done in water and in fuels, false readings can result because of extracting some of the weight of the filter which will counteract the dry weight.

Another membrane manufacturer mentioned the inclusion of the wetting agent, which is an alkylarylpolyether alcohol. This is well researched. It was selected because of low toxicity and is in the formulation primarily for pore formation and controlled pore size. The amount which is removed in the manufacturing process must be controlled so that the filter is wettable. Otherwise, filtration won't take place or nonwetting spots will result. The statement was correct, but the primary reason for it is control of pore formation in membrane production.

Subsequently, the procedure calls for removing both the wetting agent and the plasticizer in the processing.

I want to point out that during the development of the total coliform MF test, thousands of tests were run. There was a competition between the membrane filter and the MPN procedures. The problem was to get acceptance from the Standard Methods Committee of the MF method in comparison to the MPN. Hundreds of papers, many contributed by some of the researchers here today, were written before this test was accepted, first as a tentative standard and then as an alternate to the standard MPN test. Subsequently, however, when the fecal coliform test was developed as a better sanitary indicator, something slipped in. It was assumed that because this was also a coliform test, the same filter should be the right product for the test. Other aspects of this test were examined. Recently, reports were published that observed a difference in recoveries in the fecal coliform test. Dr. Presswood's paper appeared first and other followed, but there was a lot of confusion. When you look at these reports together you find that different research found diametrically opposed results.

The ASTM committee that will be meeting here this afternoon to establish methods for evaluating membrane filters has decided that the fecal coliform test would be the optimum test for evaluating all microbiological membranes because of its sensitivity. They worked on the test procedure and modified it and as is the final step in all ASTM methods, planned a round robin study. A number of laboratories, including our own, agreed to run the test and compare the results to make sure that the test works. The results of the round-robin were rather confusing and you heard about that in the report by Mr. Davis. It was difficult to determine what had happened.

Our company began a research program to find the problem. We were looking for what we call the big red X, this confusing factor that led to what seemed to be total confusion in the fecal coliform test. Dr. Sladek's group spent a great deal of time researching the point. They found out that there can be an effect of the different factors. The question of different methods of sterilizing filters, autoclaving versus ethylene oxide versus high voltage were discussed. I think this point was just a little confused yesterday. Proper sterilization, regardless of the method, should not be a significant factor. If you leave ethylene oxide in a filter and test it, it's going to be toxic to the organisms, there is no question about it. That's what the ethylene was there for, to kill organisms. But if you properly remove the ethylene oxide, it will have no subsequent effect.

Let me re-draw the curve that Dr. Sladek showed in the presentation, because I feel that surface effects are not the only factor, but the key factor. After we found out about the surface pore phenomenon, we ran tests on a tremendous number of different pore size filters, comparing the surface pore openings and pore sizes against recoveries of fecal coliform in the test. With the fecal coliform test, there was a very clear pattern. If the ordinate is recovery shown as percent recovery or cell numbers and the absissa is the surface pore size measured with the electron microscope, then there is a very pronounced curve of this nature. We repeated the tests many, many times. Because of time limitations, Dr. Sladek showed only a few of the results, but what was shown clearly was that the .45 μm filter which we manufacture has a pore size opening which lies close to the top of this very steep curve. I will not speak about the other membrane manufacturers' products. However, a similar curve should develop for their products. If there is a slight difference in the surface pore openings from lot to lot, and batch to batch, it is a factor that has never before been quality controlled. We did not realize and no one knew that it was an important factor, so we were looking at retention pore size and not surface pore size. In the future this will change.

Slight changes in the surface pore size or pore openings could create a tremendous difference in the recoveries and this, we are convinced, is the reason why attempts to compare autoclaved packed filters with ethylene oxide packed filters showed one thing one time, and something opposite the next time. We were not careful to use filters which were the same, from the same batch or with the same surface pore size. What we are doing as a company is recommending that for the fecal coliform test we go up into this region where slight changes in the surface pore size will have very little effect in the recovery of fecal coliform. In the limited tests we have run comparing this for optimum surface pore size filter, we found a very good uniformity of results and we have found recoveries usually higher than what we are using as the control, the spread plate. That's where our technology stands at this particular time.

In this research we came up with a few other points which are worthwhile reiterating. I think Dr. Sladek mentioned these yesterday but in terms of an appropriate control for this type of work the thickness of agar in the spread plate control is an important factor. We were just casually pouring our spread plates without carefully measuring the amount of thickness and found that this was an important factor. We use a 90 mm diameter petri dish. We ran tests using different quantities from 15 ml up to 35 ml of M-FC agar and we found a recovery which looked essentially like this with a peak at about 30 ml in the dish. But if we had too little, we had low recovery so that is a factor that we are now carefully measuring for our controls.

A second factor mentioned by several people is the dilution water. Initially, the fecal coliform test was set up with phosphate buffer dilution water and phosphate buffer is toxic to fecal coliforms. It is now written as peptone dilution water and it should be corrected, incidentally, to phosphate buffer peptone water (we will talk about that this afternoon). If you have good pH 7 distilled water, peptone water is fine, but if your distilled water is off in pH, you need a buffer. This is another factor keeping your control sample count low.

To finish off, I have another point for clarification. The question was raised about the effect of vacuum on bacterial cells. Let me say clearly that the degree of vacuum has no effect on the bacterial cells. When the cells are in the water they don't know whether your vacuum pump is pulling one inch or three atmospheres of pressure. They are not affected by the vacuum unless they are on the surface of the water where the vapor pressure has an effect. So during filtration they do not know what the vacuum is and don't care. The rate of impingement against the filter is not significantly different. It is very slow regardless of whether it is high vacuum or low vacuum. At the end of the filtration, when there is no more water going through the filter, there is a blockage effect because of Poiseville's law of capillarity. The vacuum is below the filter but they again don't know to what degree the vacuum is below the filter. So unless you let the filter sit there and dry for a long time where the drying effect could kill the cells, there is no effect of degree of vacuum on the cells.

Question from Audience: Is there anything in the literature that we could read to find out all of this? I've heard you say this often, and I have heard others talk that way but have never read anything about it.

Cotton: Well, that particular point isn't worthwhile doing a research study on, because there is no possible effect. It can't happen. The cells don't know what the vacuum is. However, I don't know of literature on that particular study.

I think I have just about covered all the points that I had here, except that as a result of our information we are going to make available a filter which has a surface opening pore size which will have a retention pore size of .7 μm. I thank you for allowing me this time to speak on behalf of the membrane manufacturers. I would be happy to answer any questions.

Bordner: Mr. Cotton, you have just given us another paper which was not on the agenda but provided additional insight into filter manufacturing processes.

Winter: We are looking at a graph showing the recoveries of fecal coliform relating to the surface pore opening. One of the points in the paper was passed over yesterday. It shows that passage of microorganisms begins, interestingly enough, at 2.4 μm. This means that cells pass through your membrane. If we will take Sladek's description of the standard deviation as being the square root of the mean, and the mean pore opening size is 2.4 μm, then one standard deviation is about 1.2 μm and that means that half the pore opening sizes are greater than 2.4 μm and hence are passing cells?

Sladek: I was speaking of a certain contribution to scatter in measuring counted data, such as you have in bacteriology where you count a number of colonies. Now this is not counted data, this is a measurement which is more like a measurement of temperature, and on something like a pore distribution there is no necessity for the standard deviation to have any particular size. That is, there isn't a **Poisson** distribution involved. This is not a counted sort of phenomenon. In fact, the width of the pore distribution in membrane filters is controlled entirely by the detail of the manufacturing process. It is considerably narrower than what you were just saying.

Winter: Well, how narrow is it? This is a point that has always bothered us. In any of the literature, manufacturers talk about .45μm openings. They show neat pore size distribution in their literature which has no tails. I have always been curious as to how they do something that nobody can do, which is to cut off the tails of the distribution. It shows that as you go up in pore size opening on the surface, you are also enlarging the size of the internal pore so that at 2.4 μm the internal pore size is not .45 but 0.7 μm. So pores are coming very close. Are we retaining all of the cells?

Cotton: May I speak about that question since I'm the person that orginally cut off the tail of that curve. That curve comes from work in measuring pore size with the mercury intrusion technique. I won't go into detail be-that takes quite a while, but there is a tail on both ends of that curve and that was cut off for the purpose of explaining our case. The key factor as Karl pointed out yesterday, is retention of particles which are filtered through the filter and the criteria for the .45 μm filter as established by the government was 100% retention of **Serratia marcescens** cells which average in size of .6 to .7 micrometers in diameter. The coliform cells that we know about, and as described in Bergey's manual are all larger than that. I think Bergey states 1.2 μm to 2 μm. They may expand on the range, but our experience is with filters which average this size and in which there is no passage. That is, the filters made at the optimum size in the middle of this curve showed no passage whatsoever. We began to see passage at the 3 μm size, so if you look carefully at the graph we showed you will see some passage at the 3 μm surface pore opening and this would answer the spread question also. The passage is probably from the larger pores in that particular filter. Its not that the precise size shows no passage, but it's the filter at that pore size that has pores larger or smaller which show no passage.

Bordner: Are there any questions related to Dick Cotton's comments?

Seidenberg: Do I understand that you are developing another membrane with a surface pore size which will be suitable for fecal coliform?

Cotton: Yes. Developing may not be the proper term, because we can now produce membranes with any graded pore sizes. Let's say we have another membrane which we are going to make available.

Seidenberg: Does this mean that we'll have to have one membrane for total coliform and another for fecal streptococci?

Cotton: Not necessarily. It is our current feeling that the filter which we are going to recommend for fecal coliform will be completely satisfactory for total coliform, although not necessary for total bacteria count. As for fecal streps, we can't answer your question just yet because we will have to evaluate that parameter.

Seidenberg: Then it's possible that when we do tests we may have to use one of more different types of membranes, is that right?

Cotton: That's possible.

Seidenberg: That's more confusion. Thank you.

Cotton: This is true, of course, with a great many of the procedures even now. There are a variety of membranes available; you may just be concentrating on the one .45 μm membrane, but there are not 14 different pore size membranes.

Seidenberg: I'm only thinking of bacteriology.

Cotton: For the optimum test, it may be necessary to use different filters.

Bordner: Dick, can we review? Is this new membrane with the wider surface pores, the HC experimental membrane?

Cotton: At the present time, our company is calling this the HC experimental membrane.

Bordner: Is this a membrane that has an approximate mean pore size, if we can use those terms, of 2.4 μm on the top surface and .7 μm in the bottom which we can call the retention pore size?

Cotton: Surface pore size of 2.4 micrometers, plus or minus, we don't know exactly yet, and a retention pore size of .7 micrometer.

Brodsky: I wanted to ask whether Millipore or other membrane filter manufacturers in their quality control work on membranes have looked at the filtrate to see the rate of passage of the organsims through the filter. I discussed this, this morning with somebody, and I think it is a valid point. When you show on this point of the graph that any slight variation in retention pore size can have a tremendous effect on surface pore size, and we know that some organisms are always going to pass through, they don't always line up in any particular direction; they don't polarize themselves, so that you get an increased loss of organisms through the filter due to slight variations in surface pore retention size.

Cotton: Let me clarify that point. Passage of organisms through the filter is a very important factor with us. We do check very carefully to determine whether or not the organisms have passed through, both in this test and more significantly in sterile filtration procedures. Passage test data were presented on the graph that Dr. Sladek showed, and all filters with the larger pore size-surface pore openings were checked for passage. This explained to us why we got a tail-off on this curve, it was due to passage. That passage data was presented here and if you got a copy of Dr. Sladek's paper I think it is explained more clearly. What we found was that at the optimum surface pore size, there was no passage. Actually, we got just as high a recovery with larger surface-pore size, but because of passage, the recovery dropped. If you added the passage data to the recovery data you came out about the same until you reached the point where the pores were so large that media transports were excessive and you got sloppy colonies, spreading and difficult-to-read colonies. So that was really the upper level, except for the passage factor which was carefully studied.

Bordner: Thank you, Dick. Because Mr. Cotton is from Millipore Corp., I feel a need to invite comments from other MF manufacturers. If none, we welcome any additions or comments on this summary.

Litsky: You know, there is an old trick: If you want to keep a guy quiet you make him a moderator. As long as I am not a moderator, I'm going to talk. I can't help thinking that this is the first time I have seen so many big-wigs from EPA in one room and I am going to take the opportunity to remind them what they want to forget. Years ago we woke up one morning with a book published by the APHA and sanctioned by EPA which required everyone to use a fecal coliform MF test. We could not fight this because if you ever went to college you learned that you follow what the coach believes and the coach in this case accepted only fecal coliforms. It was pushed down our throats without proper evaluation because, if we had had time to evaluate we would have found the test did not work for all types of samples. Most of the fault lies with EPA; however, we in the universities and in the other non-federal laboratories are at fault because microbiologists are very lazy people. When someone publishes a method which gives two steps, we accept it without question because 1) the coach accepted it and 2) no one had the guts to question it. I was a little teed off with Presswood because we were content. We were very comfortable although we knew it wasn't such a good method, but EPA wanted it and it saved us some time and money, so we accepted it. I hope to God we don't repeat this mistake.

We (Litsky, Rose, and Geldreich) proposed a method yesterday. We proposed the method only to ask all of you people to try it and tell us whether we are on the right track. I think we are trying to make a point. I think we all look to EPA for guidance and this is the way it should be; however, I hope EPA takes the warning that we are not going to accept any other methods without the proper round-robin testing, the proper field testing and without the proper data to prove that the method proposed is better than the method we have now. I hope EPA takes the lead because I speak for all the private, city, and state laboratories. We are anxious to cooperate. Use us, but don't abuse us.

Bordner: Thank you Dr. Litsky. I feel that not only have our minds been stimulated but also, at least psychologically, that area of anatomy that young children usually have stimulated after getting into trouble. It occurs to me that EPA has always tried to work through the concensus of opinion and through Standard Methods of which you and several others within and outside of EPA are members. So we try to protect the Agency from single interest pressure. We will accept the guidance and, I hope, the help of interested microbiologists, academic and non-government. Are there other additions or critiques on the summary?

Hendricks: I really don't know what to say after Dr. Litsky's comments. Certainly I'm not an EPA big-wig. I'm big, but the stature and the agency position do not correlate. I would like to say to Dr. Litsky that his comments are true in many respects. However, I would say this about the fecal coliform tests. They have served us well. We are fortunate that we have reduced the intestinal disease rates in this country and other parts of the world where this problem has existed.

There is no doubt that we can do better and I would like to make three comments. One is about the organisms which are used in tests to evaluate media, filters and culture procedures. We all know that when we grow a pure culture in a broth and recover it under stress conditions, whether the stress is temperature, nutrient concentration, or inhibitors, the results will be low. Organisms that you introduce in the environment become stressed by environmental parameters as Drs. McFeters and Stuart have shown. To a great extent we are going to have to control the way we use our organisms, whether they are pure or natural cultures, if we are ever going to achieve uniformity in the procedures that we have been talking about this week. There is plenty of data to demonstrate this. We need a standard way of treating our cultures.

Secondly, the papers of Presswood and Brown and others, I think show quite clearly that there is a problem with membrane procedures. Let's look at the reasons why these results occur. I think we have had enough of the observations.

Thirdly, Ed Geldreich mentioned yesterday that there can be one coliform per 100 ml in drinking water. So if we recover additional coliforms by improved procedures, what is this going to do to our standards? We should keep in mind the general significance of an improved technique. Something has to be done with the data and we had better know what we are going to do with it once we get it. The concept that recovery may be a function of survival is a valid one.

Fourthly, I am aware of a paper by Kerr, University of Georgia, that showed visual differences by electron microscopy in Millipore, Nuclepore, and I think, Sartorious membranes. This technique might be of value to those of you who want to observe bacteria after culturing by membrane procedures. Thank you.

Bordner: I might add that Dr. Kerr was invited to give a paper at this symposium and would have accepted had he had the travel funds.

Power: I would like to just make a comment or two. We had this discussion yesterday on media. The point of the discussion was to tell you that we have specifications for all our media and that we use the best ingredients we can obtain. Speaking for my own company, we are not a chemical manufacturer. We buy many of the ingredients from reputable companies. On receipt, we do quality control tests to insure that the product is what we ordered and what it says on the label. Just as the user can take a good product and ruin it in the preparation, we are capable of taking good ingredients and ending up with a product that doesn't perform. That's the purpose of the quality control laboratory. We do not release any product that we feel won't perform for the purpose for which it was intended.

From the discussion, I understand that some of you have had problems. I personally have handled in my department all product reports for four of the last six years. I am not aware of any great number of problem products so I would ask that you would let us know if you feel that you are having a problem. This can be done in several ways. If you ever see a Bio-Quest representative you can inform him. Most of you probably never do, so please call or write and tell us of the problem. I will send you an authorization form to return the product. A tremendous amount of material comes to our receiving department and to insure that it gets into the right channels I would like to provide an authorization form. We would like to have return goods for testing. If that's not possible, at least provide the lot number. Then I can check the production record and see if there was anything in there that would indicate that the product might be any different or any suspicion that it has deteriorated. I can't do anything without the lot number.

If I can get returned goods we will set up an evaluation with our reference shelf sample of the same lot and the current lot and see the results. We may be able to find the problem. If we don't know about it, we can't do anything.

You are paying good money for the product and we are putting a lot of effort into them. There is no sense for anyone not to use the best available. We can make changes. We are making media according to established formulations of course. We are here to serve you. We hope to give you a degree of stability and a lot-to-lot uniformity and to take out some of the variables. I would encourage you to contact us. Of course, one of the problems is turn-around time, but I will inform my people that if any reports come in on these types of products to please let me know. I don't handle every one of them individually any more. I will try to get you an answer as quickly as possible.

There are some comments that I have heard about the storage problem. If the medium starts to cake, you have moisture and can have deterioration. So I encourage the purchase of small quantities, tight covers and the use of a dessicator particularly for small bottles. I'll go back and see if there is anything that we can do as far as stability.

Someone said that if they reorder there is a long lag time before they get the product. This is a problem. It could be our fault through poor scheduling. It could be a problem due to lack of availability of some ingredients. We have made an effort in the past year to improve our scheduling and cut down on the backorders, but I know it is a problem at times. I would encourage you to call my office or write if you prefer. If you will keep in touch with us we will do all we can to help you. All of the phone calls, letters, product reports, etc. of a technical nature come to my attention. We are there to help you and you can take advantage of it. Thank you.

Winter: I have a question for you, Dave, and for Aaron Lane, if he is here, EPA is now coming out with its own manual of methodology which includes a section on quality control. In it we are urging a limited holding time for media and one of the problems is that we can't get the manufacturers to put a date on the bottles.

Power: By law, we do have a date on them. There is an expiration date and I don't have the figures with me but I would imagine it is two years on these dehydrated materials.

Winter: Do you have a list of recommended times for holding different media? The recommended holding times differ with each media. Some, of course, like lactose broth are pretty stable. Is this list available, I think it would guide the labs. One problem with some laboratories, particularly state or federal laboratories, is the need for unique type media which they buy in quarter pounds and use only once in a year.

Power: The stability and expiration date that we put on will be for the unopened bottle, because after it is opened, I don't know. The more it is opened the faster it is going to deteriorate. I don't think that we can say, we have to have data to show that a bottle of unopened medium is good for a given time period and expiration date. Once it is opened, each bottle is unique and stability depends on whether you are in a humid climate or a dry climate. Of course the product has been stored at a distributor; also it may be stored in your facility and then stored in your lab before its opened. The user opens it, some put the cap on tight, some put it on loose and some put it on crooked; so there is no way that we can control it. I think in some cases the criticism is somewhat unjustified because we feel the product that we send out is okay. So what happens after that? We have not had expiration dates until this year. As of September 15, under the new FDA regulations, every newly manufactured product should have an expiration date and every product must have a lot number. Perhaps you can come up with some guidelines or some help from that standpoint. I don't think that we can.

Winter: You are saying that your responsibility essentially ends when the product leaves your plant, but that we could supply guidelines for storage.

Power: In your manual, you could certainly use guidelines. We have enough to do to justify the dating. Perhaps we are talking abour relatively few products of interest to the people here. Our company and our competitors are talking about thousands of products. Once the bottle is opened, everybody is going to handle it a little differently. I have no objections to anything you might want to say about storage or handling media in the laboratory. We are only going to take it up to that point and verify how long it should be good unopened.

Winter: Could you provide the data on your recommended guidelines?

Power: Yes.

Bordner: Could we have the comments from the other media manufacturers? Would you gentlemen mind remaining up front for a minute because I think we might have questions related to media.

Lane: Concerning Dave Power's paper, the comment was made this morning that there is a shift in responsibility of quality assurance from the manufacturer to the user. I don't believe that was the intent of the paper. Certainly, we don't ask the user to do all the quality assurance testing of a culture medium. Both the manufacturer and the user must have a quality assurance program. Our quality assurance, as Dave pointed out for his company, stops when the bottle leaves our plant. Every component of the culture medium - protein source, carbohydrate source, buffer, indicator, selective agents - is tested alone and in combination. The complete medium is tested for productivity, pH, and appearance when it is bottled. Then the bottle is sealed, capped and shipped to your laboratory.

The user has responsibilities for the media. Before you use it, you must make sure that the medium is placed into a refrigerator if the label so states. When the medium is delivered into the laboratory the date of receipt and the date that the bottle was opened are both on the labels. Don't open the bottle until the previous lot has been used up. That will give you a longer period of use. Store the medium at temperatures below 25 C in a low humidity environment and out of direct sunlight. Do not store the medium near an autoclave or drying ovens.

When you weigh the medium, use a balance and weights which are frequently checked for accuracy. Do not weigh in a draft or high humidity area.

Do not leave the dehydrated medium exposed to the air. Get it into solution quickly or it can harden, then promptly return the cap to the bottle and tighten securely. Return unused portions to their proper storage. Most dehydrated media are very hygroscopic and the ingredients may be sensitive to excessive moisture and light and heat. Exposure to such conditions, especially when the cap on the bottle is not securely tightened, may result in moisture uptake which alters the physical, chemical and bacteriological properties of the medium. The result could be hardening of the freeflowing powder, darkening of the powder, oxidation of some of the components, change in pH, change in solubility, change in the appearance of the color of the dissolved medium and reduction or loss of productivity, selectivity or differential characteristics.

Dissolving of the medium is another point. If you use glassware that has not been thoroughly washed, residual detergents may cause low counts. Dirty or improperly washed glassware can change the color of a medium, increase or decrease in pH, cause a precipitate reaction between the residue on the glassware and the medium component, or produce toxicity from residual detergent.

Impure water or water which has been stored in a soft glass bottle or exposed to the laboratory air, can easily alter the quality of the medium. A good distilled water which has been in a bottle on the shelf for two or three weeks with a lose stopper will take up CO_2 and that, too, can effect the pH of the medium. It can yield a precipitate in the medium or impart toxicity.

Test the water that you are using to dissolve the medium for conductivity, metals and other inhibitory substances. A pH of a good distilled water is usually between 6 and 6.5. Do not use water suspected of containing chlorine, copper, lead or detergents. We have run into this problem when we had complaints of culture medium. We would send a man to check how the distilled water was made. In one instance, we found distilled water being made in a copper still.

Avoid scorching the medium on the bottom of a flask or other container by stirring during heating; and if you are heating an agar medium be very careful. Distribute the medium uniformly and dissolve completely. To avoid overflow of the medium, do not prepare media, especially agar, in a flask less than 2½ times the volume of the medium. Prepare only sufficient quantities suitable for use in a week or less.

Do not attempt to readjust the pH of a medium unless you have proper equipment and know the procedure. Adjusting the pH of a broth is not really difficult, but adjusting the pH of an agar is quite difficult. The final pH of a medium after autoclaving and cooling to 25 C is on the label. A medium prepared according to directions in distilled or deionized water and which has not overheated during dissolving and has not been over autoclaved should have that pH.

Be sure that the temperature and pressure gauges on the autoclave are accurate. Careful timing during autoclaving is essential. Remember not to start timing when the steam starts entering the autoclave. Avoid under or over-autoclaving, especially over-autoclaving. Frequently check the efficiency of the autoclave with a biological indicator such as **Bacillus stereothermophilus**. Do not keep a sterilized agar medium in a water bath at 50 C for more than 45 to 60 minutes. The agar can settle out and phosphates can precipitate. Do not autoclave a medium containing heat labile enrichments or additives which precipitate by heating. Since heat penetration is slow in culture media, especially media containing agar, it is important that the recommended sterilization period be strictly adhered to. The time required to autoclave a medium depends not only on the efficiency of the autoclave but the volume of medium in the bottle and its size and shape. Over-autoclaving a medium, especially an agar medium, can cause: development of precipitate, change in pH, carmelization or darkening, depolymerization of the agar and reduction in gelation, reduction in productivity, selectivity and increase in inhibitory substances.

After the preparation, some media can be left at room temperature in screw-capped tubes. Agar plates must be in sealed plastic bags, preferably in the refrigerator. As Dave pointed out, the best prepared dehydrated medium can be destroyed if it is not handled properly, so I do take exception. I don't believe that the manufacturer of dehydrated media wants to shift the responsibility of quality assurance to you. You are part of it.

Bordner: Aaron, would you be willing to share that material that you read with us?

Lane: This is a quality assurance manual that we recently completed. It is available. We can send it to you at any time.

Power: In the transcript of the paper I gave yesterday is a reference to a paper published a couple of years ago by Dr. Vera who at the time was head of quality control laboratory. It says very much the same thing that Mr. Lane read. If you write to us we will send you a copy of our manual as well.

Geldreich: The points you are making are not new. We have evaluated state laboratories for over 15 years. We have trained state people who in turn evaluate the laboratories examining public water supplies throughout the state. The laboratory certainly has an important responsibility in this area.

Once a medium leaves the manufacturer there are lots of things that can go wrong with it. I recently had one laboratory throw out over $200 worth of media which was caked. We write strong reports on these subject matters. If we don't have standardization or control over media, the results will be meaningless. For instance, one laboratory in Hawaii reported that they couldn't get coliforms to ferment brilliant green when they used it as normally prepared, but of they doubled the concentration of the brilliant green they could get gas production. These things do happen. Through laboratory evaluation, EPA will encourage the people in the laboratory to maintain a good quality control program. That's their responsibility.

However, there is another form of quality control where manufacturers can help. We know there are bad batches of media. This generally is what happens: the laboratory calls the representative of the scientific supply house; the representative comes in and replaced the old batch with a new lot of medium. However, others may have been using that bad lot of medium. We don't know its bad unless we have quality control check it. I would urge the manufacturers who find a bad lot or batch of media to recall it so that the rest of us don't have to find this out the hard way. In New England where two states next to each other had the same lot of medium they had the same problems with it. The company recalled the medium, but I know of another state close by that used that medium. They didn't quality control it so they consumed the batch of medium. I am asking you and others who manufacture media that if once you find that you have a bad batch tell us you will replace it or refund our money. Look up in the records and find where in the market the product is being used and recall it. Thank you.

Bordner: Aaron, do you want to reply to this comment?

Lane: I'm quite sure that our quality assurance laboratories do not approve a batch of medium unless it performs in the manner for which it was designed. With the coliform MF media as I mentioned yesterday we use river water and ATCC cultures. I am not saying that those state laboratories mishandled the medium. Something may have happened. The problem is that the manufacturers are not being informed. If we were given the opportunity to test the sample that the state is using against our official sample, there may not have been a problem. Our quality control laboratory must report all problems and complaints they are made aware of. Did these state laboratories talk with our representatives?

Geldreich: Yes, I am told they did. I was not there. The man gave them a new lot and took the old lot away.

Lane: I would just like to say that many of the products are purchased through distributors and in the case of performance complaints I would ask that you come back to the manufacturer. You are probably visited by distributor representatives far more often than you are by our own sales force. In such instances, I don't know whether the reports get back to us. I do get letters once in a while from one supply house in particular.

They have a form letter which is very helpful. I think you should talk with the manufacturer. If you have other problems, deal with the distributor - he's the one you brought it from. When you have performance problems which are serious, deal with the manufacturer. If you want to call, that.s fine. We will take it from there.

Without the lot number, one doesn't know how many years that product has been sitting around. I know some of the distribution houses have not rotated their stock. I think that is another advantage of the expiration date. I know that once in Dallas, I started to get reports of people receiving material that was far too old. It was traced back to one distributor who had found a box that he didn't realize he had and shipped it all out. In the future this should improve. If on a re-test we find that the reference shelf sample has deteriorated prior to the expiration date we would be obligated to do a withdrawal. We know to whom we shipped it. It's up to the distributors also to have records to whom they shipped the material. I think we have a fairly good chance of getting back through the distribution system. Anything that can happen, will happen, sometime, somewhere. We must realize that we are dealing with variables in biological materials and transportation system, laboratories, samples and technicians. We will do the best that we can, but I would encourage you to come back and talk with us. At least there is a chance that it will be recalled.

Bordner: The key word is communication, right?

Alico: Its been a fine symposium and I want to offer my gratitude to the two people who summarized the presentations. I have suggestions on the title of the symposium. It reads: "The Symposium on the Recovery of Indicator Organisms in Applying Membrane Filters." I would like to add "For Coliform and related Organisms" since the general papers, the stress papers, and comparative papers dealt with coliform-related organisms. I suggest this so that when we get the proceedings of the symposium it will be clear that it deals with this one aspect of membrane filters and not with others.

One other comment I have is about the Nuclepore membrane filters. I believe about a year and half ago at the ASM meeting in Miami, I received information from the people at Nuclepore on using these filters for the enumeration of microorganisms. It was stated yesterday that they should not be used in enumeration of microorganisms. I think Nuclepore should notify people that their product should not be used for this purpose.

Harris: I think what I have to say is germane to the issue. The last speaker from EPA slipped in a little statement which I think is the whole reason for our being here. He made the rather astounding statement, in my view, that gastrointestinal diseases were on the wane. This is just not so! The incidence of salmonellosis in North America, I'm talking of the whole continent now, is rapidly on the increase. We are talking of notified cases. Bear in mind that the average physician does not notify. Probably only one in ten is notified. So which ever way you slice the cake the reason for our being here is not simply to design a system. Our basic reason for being here is the eventual reduction in G. I. diseases. If we take this as our measure, we can fail, ladies and gentlemen, fail dismally. I think that it is important that we do design a system, whether it is the fecal coliform indicator system or not. I'm speaking as a microbiologist and as a physician. I'm seeing both sides of the issue. I know what happens from the physician's side of the fence. He is presented with a lot of data. He is told thus and such an incidence of such and such an organism. He has not been trained. Even many of our physicians in the Public Health field have not been adequately trained to interpret the laboratory data.

We have been very smug as scientists in designing indicator systems, coming up with good systems for protecting public health, but leaving it there. We have not taken the trouble to bridge the gap between the scientist and the physician. I think that we should not just be satisfied in designing a system but also make sure that there is some carry-over from this type of meeting to the people who are going to go into the field and implement our findings. Unless we do that we can sit here for the next 20 years designing better and better indicators but not getting to the root of the problem which is the reduction of G. I. diseases. I would like to correct the statement made by a gentleman from EPA, that G. I. diseases are on the decrease.

I wanted to speak before the gentleman from BBL and Difco got their plug in. Maddox Chemicals supplies the Oxoid media in Canada exclusively. I go along with large measure with what Dr. Lane said. Many of faults are at the user level. I am not saying that the media manufacturers are faultless. But many of you who use Oxoid media are aware that if you run into problems we send people down. I would underline that we don't do anything about a bad product or bad use of a product unless we know something about it. Thank you.

Bordner: We are glad to have the opportunity to exchange ideas - and problems - with the media manufacturers at this symposium.

Hendricks: First of all, let me be the first to applaud the physician from Canada who took exception to what I had said about the incidence of intestinal disease. I think it is true that there is an increase in terms of "running rampant" is a relative term. I consider venereal disease in this country to be rampant, but whether this is due to reporting, or increased actual number of cases, I don't know. I rather suspect that it is a combination of the two. I will stand by what I said earlier that where the coliform tests are used to monitor water quality, I think the rate of serious intestinal disease is much lower than those areas that do not employ such procedures. Of course, these microbiological procedures have to be followed with adequate treatment of some sort. I do believe that they have served us well. The mechanisms by which intestinal disease seems to be increasing undoubtedly is due to a variety of things. I would hate to see us abandon our coliform procedures, to say that they are no good and throw them out, because I think the results of not monitoring water quality would be disastrous. This is really what I was saying about the significance of any technique that's going to have a tendency to increase numbers. We know that our measurements of coliform procedures may at times be low. This may very well be one of the reasons why we can recover pathogens, bacterial pathogens including viruses in water that appear to be of excellent quality. Again, I applaud the gentleman, but I think there are two sides to the picture and we should not eliminate procedures that have served us well. I think we should improve upon them and be well aware of what we are counting. Thank you.

FINAL REMARKS

Frith: I think everyone agrees that this was a very timely conference and we had an opportunity to share some of our general knowledge as well as to open up some additional avenues for in-depth study. I don't think that anyone will leave thinking that they got short-changed from this meeting. It is important to understand the function, and I don't want to give you a long detailed and boring explanation of ASTM but as your co-chairman for the sub-committee I would like to explain the real function of ASTM in this whole operation and ask you or invite you to become a participant.

It is obvious that the only way to eliminate confusion is to develop a test method that will help everyone to know that they are buying a standardized product, that will represent the state of the art. For about three years, ASTM has been trying to do this with just the membrane filter, not the media - nor the various controls. We have written three draft procedures: one on recovery that you have heard about today, one on inhibitory effects and one other being proposed today on retention. This afternoon we will be working on two or three of these procedures that have been round robin tested, from which you have seen some of the data. We are trying to eliminate some of the confusion.

As an ASTM member, you become a voter, a person who has a chance to see the draft copies and to submit negative ballots if you feel that there is something wrong with the way the technique is being run or if you have data that does not support what is being presented. These negative ballots or the additional data supplied have to be overridden to either prove or disprove your point.

Active membership and voting membership is available by joining the ASTM Society. You are not restricted from attending any of our meetings as a non-ASTM member. You can come, you can participate, you can offer data and comments. ASTM would like to see you become an active member and be involved with us. We hope not only to develop specifications for recovery, inhibitory effects and retention, but also to consider extractables, surface characteristics and other general topics such as membrane composition and impurities. You can see the charter that we have ahead of us is quite broad.

We will be doing the same thing, I am sure, with various types of media. We will be determining how you actually monitor a temperature of 44.5 + 0.2 C. All of these issues will be coming out of ASTM in the next few years. Your active participation can help that move faster. Dr. Litsky has said, "Let's either write the specs by the state of the art and make them meaningful or forget it." I'm thankful that each of you has taken time for a day and a half to attend. I think it has been a fantastic symposium and because of your participation, we will gain a lot more and move a lot quicker than we have done in the last ten years. Again I say, you are invited this afternoon to come and listen and participate. Are there any questions about ASTM?

Ginsburg: This meeting that is coming up this afternnon, will it deal with the same problems that the symposium covered?

Frith: That is correct. We hoped to learn a lot from the symposium and to use the knowledge we have gained the last day and a half to make meaningful test methods on how do you really monitor the variations in membranes or monitor the difference in water samples, etc?

Ginsberg: This same idea, is it carried through the week?

Frith: No. The test method will only carry through this afternoon. Tomorrow, there is going to be an organizational meeting for indicator organisms. We are concerned not only with coliforms and fecal coliforms but also with different indicator organisms.

Ginsburg: What is the procedure for participating in any future round robins?

Frith: Well, you'll hear about that at the meeting today. If you as a laboratory representative would like to participate you are welcome. We want to get enough different labs around the country to get a representative sample. If you feel that your lab can spend the time (it is a very exicting program but it takes an awful lot of money and time to do what is required) you will be getting notes as a member of the committee, and you will be notified that a round robin will be taking place. Margareta Jackson, who unfortunately could not be here, is the one actually in charge of the afternoon session.

Ginsberg: Do I understand that ASTM supplies the materials or do we have to buy them?

Frith: In the first round robin we asked the membrane manufacturers and one media manufacturer to supply materials but you will make the biggest contribution which is man hours.

Vlassoff: When the people fill out these forms, won't they have some idea what's happening?

Frith: Yes. That is an ASTM form, if you have not filled out one of these, you should. We will be submitting to you everything that has happened at this symposium. How fast several of us can get together and either get these papers in some form to send out or get into a publication with which both ASTM and EPA are concerned will depend on some man hours. We will keep you informed if you leave your name and address, whether you are an ASTM member or not, by this route.

Question: If this bulletin is published, will it go automatically to ASTM members or will this be a personal-type mailing?

Frith: We are not sure right now. I think the question has yet to be resolved - how and in what form this symposium be provided to you. It will not be published until some degree of satisfaction has been reached between ASTM and EPA, as they were the sponsors. Again, many thanks for your time and your talents and we will look forward to those of you who would like to join us at 1:30 back in this room.

Bordner: I want to thank all of you for participating in this symposium - those of you who made special efforts in preparing papers and presenting them, who participated by offering thought-provoking comments, and who summarized what was said. To all of you we owe particular appreciation.

APPENDIX

The following paper intended for the Seminar was not received in time for presentation. However, it is relevant and is presented here for the consideration of the readers.

COMPARISON OF MEMBRANE FILTER COUNTS AND PLATE COUNTS ON HETEROTROPHIC AND OIL AGAR USED TO ESTIMATE POPULATIONS OF YEAST, FUNGI AND BACTERIA

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ABSTRACT

Comparison of filter and plate counts of yeasts and fungi on heterotropic and oil agar revealed higher counts were nearly always obtained with filters. Comparison of filter and plate counts of heterotrophic estuarine and marine bacteria revealed that, on the average, filter counts were 80% lower than the plate counts. These results should be considered when evaluating methods for enumeration of microorganisms in the marine environment.

INTRODUCTION

Microorganisms present in seawater are usually enumerated using membrane filters because of their low numbers. However, in the estuarine environment, generally characterized by larger microbial populations, microorganisms are frequently enumerated using plate counts. In the study reported here, Chesapeake Bay water was tested, permitting comparison of plate and filter counts for yeast, fungi and bacteria.

MATERIALS AND METHODS

Heterotrophic yeasts and fungi were enumerated using yeast medium, details of which have been published elsewhere (1). Yeasts and fungi capable of growth on petroleum were enumerated using oil agar #2, pH adjusted to 5.5 and supplemented with streptomycin and tetracycline (50 ug/ml of each), as described by Walker and Colwell (2). Heterotrophic bacteria were enumerated using the basal medium of Walker and Colwell (1). Bacteria capable of growth on petroleum were counted using a silica gel-oil medium (3). All plates were incubated at 15 C for two weeks.

RESULTS AND DISCUSSION

Heterotrophic yeasts and fungi count plates were incubated for a minimum of one week to insure appearance of the maximum number of colonies (Tables 1 and 2). The results of the heterotrophic counts compared with counts for yeasts and fungi on oil agar plates incubated for at least two weeks are shown in Tables 3 and 4. It was necessary to examine the plates periodically, after the initial three days of incubation, to avoid overgrowth with fungi.

Before plate counts were compared with the filter counts, a percent comparison was calculated between the plate and filter replica plate counts (Tables 5 and 6). These calculations indicated at least 60% comparison between replicate counts was obtained. Similar comparability was observed for results of the yeast and fungi replicate counts.

Comparison of the plate and filter counts of heterotrophic yeast showed that the highest counts were always obtained when membrane filters were used. The percent comparison was always indicative of a significantly lower count on the plates compared with the filters (Table 7). In most instances, higher counts of heterotrophic fungi were obtained using membrane filters, compared with the spread plate technique (Table 8). Half of the percent similarities were significant. The number of yeasts growing on oil agar, using the filter method of enumeration, was higher than on the oil agar plates. In only one case did the two methods give a reasonably close count (Table 9). As in the case of the heterotrophic fungi, higher counts of fungi on oil agar were obtained using membrane filters, than with plate counts, although the counts were minimally comparable (Table 10). Results obtained when plate and filter counts of yeasts and fungi on heterotrophic and oil agar were compared suggested that quite different counts were obtained by these methods and that filter counts yielded higher numbers of yeasts and fungi when estuarine and marine water and sediment samples were examined.

Comparison of plate and filter counts of heterotrophic bacteria indicated that plate counts yielded higher counts in most samples, and only a few samples gave similar results by plate and filter counts (Table 11). Plate and filter counts of bacteria on silica gel medium were not high enough to compare results (Table 12). The filter procedure gave higher counts on silica gel oil for bacteria from sediment and from Eastern Bay water, whereas plate counts provided the best estimation of the bacterial populations in Colgate Creek water enumerated on silica gel-oil medium.

The two areas in Chesapeake Bay included in this study, Colgate Creek in Baltimore Harbor and Eastern Bay, differ ecologically. Colgate Creek is continuously contaminated with oil, whereas Eastern Bay is an oil-free, commercially productive shellfish area. The higher counts of yeast, fungi and bacteria on oil media for Colgate Creek samples can be explained. By virtue of larger populations, more comparisons of plate counts and filter counts were possible using inocula from Colgate Creek than from Eastern Bay.

Plate and filter counts of seawater collected at stations along the U.S. east coast were compared and, generally speaking, plate counts of microorganisms in seawater, concentrated using an Aminco concentrator (American Instrument Co., Silver Spring, Md.) yielded higher counts than the filter procedure (Table 13). By dividing the average filter count by the average plate count and multiplying by 100, a comparison was possible for estuarine and concentrated seawater samples. The average filter count for estuarine samples was 19.7% of the average plate count and 19.2% for the concentrated seawater. Thus, for the average of the 14 estuarine samples and 10 marine samples studied, filter counts were about 80% lower than the plate counts.

Nuclepore (General Electric, Pleasanton, Calif.) filters were compared with Millipore (Millipore Filter Corp., Bedford, Mass.) filters by scanning electron microscopy (Todd and Kerr (4)), but not for efficiency in enumeration of bacteria. Nuclepore filters are thin (10 um) films of polycarbonate plastic, with an average pore diameter approximating the individual pore diameter. Millipore filters are thick (150 um) films of cellulose, the average pore diameter differing significantly from the individual pore diameters. Millipore counts were compared with Nuclepore counts. Data for the estuarine samples showed no trend toward higher counts for Millipore filters in comparison with Nuclepore filters (Table 14). This was unlike the marine samples which always gave higher counts with Millipore filters (Table 15). Two distinct disadvantages of using Nuclepore filters in laboratories aboard ocean research vessels are that Nuclepore membranes are markedly thin, making it difficult to place them on sintered glass filter holders and on agar surfaces. Air bubbles trapped between the agar and the filter are often indistinguishable from translucent bacterial colonies.

As a general statement, membrane filters are more efficient for counting yeasts and fungi but plate counting for estuarine and marine water bacteria is recommended over the membrane filter method. The ease of use and their sturdier nature make Millipore filters preferable to Nuclepore filters for field work.

ACKNOWLEDGMENT

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Table 1. EFFECT OF INCUBATION TIME ON FILTER COUNTS OF HETEROTROPHIC YEASTS

		Counts for			
Incubation time (days)	Dilution/Volume filtered (ml)	Colgate Creek sediment	Colgate Creek water	Eastern Bay sediment	Eastern Bay water
3	0/100		1		1
	0/250		3		3
	-2/10	2		<1	
	0/100		2		2
	0/250		OG[a]		3
	-2/10	2		<1	
14	0/100		2		2
	0/250		OG		3
	-2/10	2		<1	

[a] Overgrown with fungi

Table 2. EFFECT OF INCUBATION TIME ON FILTER COUNTS OF HETEROTROPHIC FUNGI

		Counts for			
Incubation time (days)	Dilution/volume filtered (ml)	Colgate Creek sediment	Colgate Creek water	Eastern Bay sediment	Eastern Bay water
3	0/100		5		2
	0/250		9		12
	-2/10	2		<1	
7	0/100		10		5
	0/250		OG[a]		19
	-2/10	11		3	
14	0/100		10		5
	0/250		OG		19
	-2/10				

[a] Overgrown with fungi.

Table 3. EFFECT OF INCUBATION TIME ON FILTER COUNTS OF YEASTS ON OIL AGAR

Incubation time (days)	Dilution/volume filtered (ml)	Counts for Colgate Creek sediment	Colgate Creek water	Eastern Bay sediment	Eastern Bay water
	0/100		<1		<1
3	0/250		<1		<1
	-2/10	<1		<1	
	0/100		<1		1
7	0/250		OG[a]		2
	-2/10	<1		<1	
	0/100		5		1
14	0/250		OG		2
	-2/10	1		<1	

[a] Overgrown with fungi.

Table 4. EFFECT OF INCUBATION TIME ON FILTER COUNTS OF FUNGI ON OIL AGAR

Incubation time (days)	Dilution/volume filtered (ml)	Counts for Colgate Creek sediment	Colgate Creek water	Eastern Bay sediment	Eastern Bay water
	0/100		0		0
3	0/250		2		0
	-2/10	<1		<1	
	0/100		2		3
7	0/250		OG[a]		6
	-2/10	3		<1	
	0/100		OG		3
14	0/250		OG		6
	-2/10	3		<1	

[a] Overgrown with fungi.

Table 5. COMPARISON OF REPLICATES FOR DUPLICATE PLATE COUNTS OF CHESAPEAKE BAY BACTERIA

Bacteria/ml Replicate 1	Bacteria/ml Replicate 2	Percent comparison
5.2×10^5	4.7×10^5	90.4
1.7×10^4	2.7×10^4	62.9
6.4×10^2	1.0×10^3	64.0
1.4×10^2	1.2×10^2	85.7
2.3×10^3	1.9×10^3	82.6
7.3×10^4	7.1×10^4	97.3
3.0×10^1	4.0×10^1	75.0
2.8×10^2	2.0×10^2	71.4
4.3×10^4	4.2×10^4	97.7
1.7×10^3	1.5×10^3	88.2
1.0×10^5	1.6×10^5	62.5

Table 6. COMPARISON OF REPLICATES FOR DUPLICATE FILTER COUNTS OF CHESAPEAKE BAY BACTERIA

Bacteria/ml Replicate 1	Bacteria/ml Replicate 2	Percent comparison
30	20	66.6
15	10	66.6
33	25	75.6
44	37	84.1
51	39	76.5
45	42	93.3
50	41	82.0

Table 7. COMPARISON OF PLATE AND FILTER COUNTS OF HETEROTROPHIC YEASTS

Inoculum	Source	Yeasts/ml		Percent comparison
		Plate	Filter	
Sediment	Colgate Creek	3.0×10^1	1.7×10^2[a]	17.6
Sediment		5.0×10^0	1.0×10^1	50.0
Sediment		5.0×10^1	2.0×10^2	25.0
Water	Colgate Creek	1.0×10^0	1.7×10^1[b]	5.8
Water		$<10^0$	4.0×10^{-1}[b]	N.D.[d]
Water		5.0×10^0	1.0×10^0[b]	20.0
Sediment	Eastern Bay	$<10^1$	5.0×10^0	N.D.
Sediment		$<10^1$	$<10^1$	N.D.
Sediment		$<10^1$	$<10^1$	N.D.
Water	Eastern Bay	$<10^0$	1.0×10^{-2}[c]	N.D.
Water		$<10^0$	5.0×10^{-3}[c]	N.D.
Water		$<10^0$	2.0×10^{-2}[c]	N.D.

[a] Results obtained by filtering 10 ml of a 10^{-2} dilution of sediment samples in this and subsequent tables for yeasts and fungi.

[b] Results obtained by filtering 100 ml of Colgate Creek water in this and subsequent tables for yeasts and fungi.

[c] Results obtained by filtering 1000 ml of Eastern Bay water in this and subsequent tables for yeasts and fungi.

[d] Not determined.

Table 8. COMPARISON OF PLATE AND FILTER COUNTS OF HETEROTROPHIC FUNGI

Inoculum	Source	Fungi/ml		Percent comparison
		Plate	Filter	
Sediment	Colgate Creek	1.0×10^3	2.0×10^3	50.0
Sediment		7.0×10^2	9.0×10^1	12.9
Sediment		1.0×10^3	1.0×10^3	100.0
Water	Colgate Creek	1.5×10^0	1.7×10^0	88.2
Water		$<10^0$	2.5×10^0	N.D.
Water		5.0×10^1	1.0×10^{-1}	0.2
Sediment	Eastern Bay	$<10^1$	$<10^1$	N.D.
Sediment		6.0×10^1	5.0×10^1	83.3
Sediment		$<10^1$	3.0×10^2	N.D.
Water	Eastern Bay	$<10^0$	5.0×10^{-2}	N.D.
Water		$<10^0$	7.0×10^{-2}	N.D.
Water		1.0×10^0	5.0×10^{-2}	5.0

Table 9. COMPARISON OF PLATE AND FILTER COUNTS OF YEASTS ON OIL AGAR

Inoculum	Source	Yeasts/ml Plate	Yeasts/ml Filter	Percent comparison
Sediment	Colgate Creek	3.0×10^1	5.0×10^1	60.0
Sediment		1.0×10^1	2.0×10^2	5.0
Sediment		$<10^1$	1.0×10^2	N.D.
Water	Colgate Creek	1.0×10^0	1.2×10^1	8.3
Water		$<10^0$	$<10^0$	N.D.
Water		$<10^0$	1.0×10^0	N.D.
Sediment	Eastern Bay	5.0×10^0	$<10^0$	N.D.
Sediment		$<10^0$	$<10^0$	N.D.
Sediment		$<10^0$	$<10^0$	N.D.
Water	Eastern Bay	$<10^0$	5.0×10^{-3}	N.D.
Water		$<10^0$	2.0×10^{-1}	N.D.
Water		$<10^0$	1.0×10^{-2}	N.D.

Table 10. COMPARISON OF PLATE AND FILTER COUNTS OF FUNGI ON OIL AGAR

Inoculum	Source	Fungi/ml Plate	Fungi/ml Filter	Percent comparison
Sediment	Colgate Creek	5.0×10^1	1.0×10^2	20.0
Sediment		6.0×10^1	4.0×10^1	66.6
Sediment		2.0×10^2	3.0×10^2	66.6
Water	Colgate Creek	$<10^0$	2.0×10^{-1}	N.D.
Water		$<10^0$	1.0×10^{-1}	N.D.
Water		2.0×10^0	1.0×10^{-2}	0.5
Sediment	Eastern Bay	$<10^1$	$<10^1$	N.D.
Sediment		$<10^1$	$<10^1$	N.D.
Sediment		$<10^1$	$<10^1$	N.D.
Water	Eastern Bay	$<10^0$	2.0×10^{-2}	N.D.
Water		$<10^0$	5.0×10^{-2}	N.D.
Water		1.0×10^0	3.0×10^{-2}	3.0

Table 11. COMPARISON OF PLATE AND FILTER COUNTS OF HETEROTROPHIC ESTUARINE BACTERIA

Inoculum	Source	Bacteria/ml		Percent comparison
		Plate	Filter	
Sediment	Colgate Creek	3.8×10^5	2.6×10^5	68.4
Sediment		4.5×10^6	4.0×10^5	8.8
Sediment		6.4×10^5	1.0×10^5	15.6
Sediment		2.8×10^4	3.2×10^4	87.5
Sediment		3.2×10^6	7.0×10^5	21.9
Sediment		1.9×10^5	2.5×10^4	13.2
Water	Colgate Creek	2.9×10^4	1.2×10^5	4.1
Water		9.1×10^4	6.7×10^4	73.6
Water		2.5×10^3	4.8×10^3	52.0
Water		8.1×10^4	6.8×10^4	84.0
Sediment	Eastern Bay	9.3×10^4	5.6×10^4	60.2
Sediment		4.7×10^5	2.0×10^4	4.2
Sediment		1.6×10^4	1.0×10^4	62.5
Water	Eastern Bay	2.2×10^2	3.0×10^1	13.6

Table 12. COMPARISON OF PLATE AND FILTER COUNTS OF BACTERIA ON SILICA GEL-OIL MEDIUM

		Bacteria/ml	
Inoculum	Source	Plate	Filter
Sediment	Colgate Creek	$<10^2$	3.0×10^1
Sediment		$<10^2$	3.0×10^1
Sediment		$<10^2$	1.7×10^2
Sediment		$<10^2$	2.0×10^1
Sediment		$<10^2$	1.0×10^1
Water	Colgate Creek	5.0×10^1	$<1.0 \times 10^{-1}$
Water		3.5×10^2	$<1.0 \times 10^{-1}$
Water		1.5×10^2	$<1.0 \times 10^{-1}$
Water		1.5×10^2	$<1.0 \times 10^{-1}$
Water		5.0×10^1	6.1×10^0
Sediment	Eastern Bay	$<10^2$	1.8×10^2
Sediment		$<10^2$	5.0×10^0
Sediment		$<10^2$	$<1.0 \times 10^{-1}$
Sediment		$<10^2$	$<1.0 \times 10^{-1}$
Sediment		$<10^2$	$<1.0 \times 10^{-1}$
Water	Eastern Bay	$<10^2$	9.0×10^{-2}
Water		1.0×10^0	$<1.0 \times 10^{-2}$
Water		$<10^0$	$<1.0 \times 10^{-2}$
Water		$<10^0$	7.5×10^{-2}
Water		$<10^0$	5.0×10^{-2}

Table 13. COMPARISON OF FILTER AND PLATE COUNTS OF HETEROTROPHIC MARINE BACTERIA

	Bacteria/ml		
Inoculum	Plate	Filter	Percent comparison
Sea water concentrate	3.4×10^5	4.8×10^5	70.8
Sea water concentrate	1.5×10^5	5.0×10^4	33.3
Sea water concentrate	1.8×10^6	1.8×10^5	10.0
Sea water concentrate	5.0×10^5	1.4×10^5	28.0
Sea water concentrate	9.0×10^4	7.4×10^4	82.2
Sea water concentrate	2.0×10^6	5.6×10^4	2.8
Sea water concentrate	3.0×10^5	6.0×10^3	2.0
Sea water concentrate	2.6×10^3	7.1×10^1	2.7
Sea water concentrate	6.2×10^2	7.2×10^2	86.1
Sea water concentrate	1.1×10^4	3.5×10^4	31.4

Table 14. COMPARISON OF FILTER COUNTS OF ESTUARINE BACTERIA OBTAINED ON MILLIPORE AND NUCLEPORE FILTERS

		Bacteria/ml		Percent
Inoculum	Source	Millipore	Nuclepore	comparison
Sediment	Colgate Creek	2.6×10^5	2.8×10^5	92.8
Sediment		4.0×10^5	6.0×10^5	66.6
Sediment		1.0×10^5	1.0×10^5	100.0
Sediment		3.2×10^4	3.2×10^4	100.0
Sediment		7.0×10^5	1.9×10^6	36.8
Sediment		2.5×10^4	1.0×10^4	40.0
Sediment		20	8	40.0
Water	Colgate Creek	1.2×10^5	7.9×10^4	65.8
Water		6.7×10^4	8.3×10^4	80.7
Water		4.8×10^3	3.4×10^3	70.8
Water		6.8×10^4	5.3×10^4	77.9
Sediment	Eastern Bay	5.6×10^4	7.4×10^4	75.7
Sediment		30	30	100.0
Sediment		2.0×10^4	1.0×10^4	100.0
Sediment		16	2	12.5
Sediment		1.0×10^4	1.2×10^4	83.3
Sediment		6	12	50.0
Sediment		3	3	100.0
Water	Eastern Bay	3.0×10^1	1.0×10^1	33.3

Table 15. COMPARISON OF FILTER COUNTS OF HETEROTROPHIC MARINE BACTERIA OBTAINED ON MILLIPORE AND NUCLEPORE FILTERS

	Bacteria/100 ml on		Percent
Inoculum	Millipore	Nuclepore	comparison
Sea water	360	340	94.4
Sea water	162	147	90.7
Sea water	260	106	40.8
Sea water	268	100	37.3